claim 41 in which a phenotypic change has been produced by activating expression of the MMP by withholding tetracycline or a tetracycline analog during adulthood of the transgenic mouse;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mouse or rat to which the composition was administered to a control mouse or rat in which expression of the MMP was activated without administering the composition,

wherein any less extensive development in the nature or extent of the phenotypic change, or any increased length of time required for the phenotypic change to develop in the mammal that has been administered the composition relative to the control mammal indicates the potential of the composition to counteract the phenotypic change.

REMARKS

This submission is in response to the Office Action dated January 30, 2002. Claims 28, 30-32, 35-38, 40-46, 48, 49, 51-54 have been amended. The amended claims are supported by the specification as set forth below and in Applicant's Preliminary Amendment filed November 20, 2000. Claims 28-54 are pending.

Reconsideration of the above identified application, in view of the above amendments and the following remarks, is respectfully requested.

SEQUENCE LISTING

Pursuant to the Examiner's statement regarding the absence of a Sequence Listing and corresponding SEQ ID NO for the nucleic acid sequence depicted in Figure 1B which represents a variant of MMP13, applicants note that this nucleic acid sequence has been provided in the Sequence Listing as SEQ ID NO:18. In addition, the pro domain and catalytic domain sequences depicted in Figure 1A are provided in the Sequence Listing as SEQ ID NO:19 and SEQ ID NO:20, respectively. Finally, the sequence for the additional MMP-13* variant wherein Val^{98} is substituted with Gly as disclosed at page 12, lines 17-18 of the specification has been provided as SEQ ID NO:21. The specification has been amended to add the identifying SEQ ID NOs to the disclosure.

In view of the foregoing, the Sequence Listing filed in this application is believed to be correct.

INFORMATION DISCLOSURE STATEMENT

The Examiner has objected to the IDS filed on February 28, 2001, stating that the citations are incomplete. However, the Examiner has acknowledged reviewing the references by initialing the PTO 1449. Moreover, these references

are of record in parent application Serial No. 08/994,689. Thus, the Applicant's obligations under Rule 56 have been satisfied. The Examiner has stated that the reference DE 19501032A1 has not been considered because a translation has not been provided. However, in the Office Action dated March 1, 2000, in the parent application (08/994,689), the Examiner indicated that this reference was considered and made of record by initialing PTO Form 1449.

CLAIM OBJECTIONS

The Examiner has requested placement of a comma between "constitutively" and"enzymatically" in the claims. Applicants respectfully decline, as the term "constitutively" directly modifies the term "enzymatically active." Consequently, there should be no comma.

The preamble change requested by the Examiner has been made.

CLAIM REJECTIONS

35 U.S.C. §112, first paragraph

1. Written Description

The Examiner has rejected claims 28-54 under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) at the time the application was filed, had possession of the

claimed invention.

Specifically, Examiner notes that adequate written description of a transgenic non-human mammal having a characteristic of osteoarthritis requires a description of the combination of DNA encoding an extracellular matrix enzyme, chondrocyte-specific promoter, and the method of making transgenic non-human mammals required to obtain a transgenic non-human mammal having a characteristic of osteoarthritis.

In response, Applicants respectfully direct the Examiner's attention to the claims, which affirmatively recite these elements. Contrary to the Examiner's assertion, these claims are directed to transgenic animals having the recited genetic structure, and which thus are capable of expressing the desired phenotypic characteristics.

The phrase "chondrocyte tissue-specific promoter" finds support in the application on page 37, line 1; page 6, lines 4-5, and page 13, lines 3-4. There is an extensive description of promoters that direct transcription in joint tissues, i.e., that provide spatial control of expression, on page 15, line 19 to page 16, line 6 of the specification. The specification clearly discloses that expression of a matrix decoding enzyme (MDE) in chondrocytes, which are the cells found in joints, results in localized degradation of extracellular matrix proteins. Having established this principle with a working example (the Type II collagen promoter), one of ordinary skill in the art would recognize that joint (i.e., chondrocyte) tissue-specific

expression of an MDE, particularly a collagen II-degrading MMP, would yield the desired joint degradation.[1] The thrust of this ground for rejection is misplaced: Applicants claim transgenic animals that have tissue restricted expression of the transgene, and the invention is exemplified with tissue restricted expression of both a marker (beta-galactosidase) and a matrix degrading enzyme (the MMP-13 variant). The Examiner provides no reasoning to support a lack of written description of the invention as a whole.

Applicants note that a sequence of an extracellular matrix enzyme is depicted in SEQ ID NOs: 1 and 21. The matrix degrading enzyme-encoding genes of the present invention are selectively expressed in articular chondrocytes of the transgenic animal and this joint-restricted expression results in degradation of extracellular matrix proteins, e.g., collagen II, leading to pathological symptoms characteristic of degenerative joint disease. Accordingly, the MDE, such as a matrix metalloproteinase specific for collagen II, is selectively expressed in articular chondrocytes and thus a "chondrocyte tissue-specific promoter" is the appropriate description for the promoter that functions specifically in chondrocytes.

The claims have been amended, *inter alia*, to recite that transgenic

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[1] This claim does not "preempt the future before it has arrived" as feared by the Examiner (Office Action, page 5, citing *Fiers v. Revel* and *Regents of the University of California v. Eli Lilly*). The future -- other chondrocyte tissue-specific promoters -- is free to arrive unhindered. However, claims of proper scope preserve to the inventors the full measure of their invention, which includes use of any such "future" chondrocyte tissue-specific promoters to generate the claimed transgenic animal.

expression can be repressed in the mammal until adulthood (in the embryonic and fetal stages) to permit development of a viable mammal, and induced in the adult to cause a phenotypic change characteristic of osteoarthritis in the joints of the adult mammal. These functional characteristics of the claimed mammal are exemplified in the patent application, *e.g.*, in example 5 (page 41-44) and example 6 (pages 44-45). Applicants have thus developed a transgenic mammal in which temporal control of transgene expression is effected by environmental influences under the control of the investigator. Such a system stands in contrast to a system in which temporal control of gene expression is determined by an endogenous temporal promoter, *e.g.*, a promoter involved in expression of developmental genes, which can switch on and switch off at different times in development of the animal. Thus, in the present invention transgene expression does not occur in the mammal through endogenous temporal expression regulation. Rather this transgenic mammal is constructed to permit the investigator to induce expression, and this feature is intrinsic to the claimed transgenic mammal.

The Examiner argues that the specification does not provide adequate written description for using any and all sequences of SEQ ID NO: 1 or 21 in the instant invention. The claims have been amended to recite that the MMP comprises SEQ NO: 1 or NO:21, thus obviating this rejection.

Next, the Examiner points out that the specification allegedly does not provide adequate written description for using any and all sequences of a tet07

sequence (claim 37), SEQ ID NO:2 (claim 38), a Type II collagen promoter (claims 39, 42) in the instant invention. Applicants note that support for claim 37 appears in claim 11 as filed and in page 14, lines 21-22 of the specification. Furthermore, claim 37 as amended to recite "the regulatable promoter comprises the tet07 promoter" further obviates the rejection, and thus the rejection should be withdrawn. Support for claim 38 appears in claim 12 as filed. The full sequence listings sufficiently describe SEQ ID NO: 2. The claim language of claim 38 was also amended to more particularly point out the invention, reciting "the regulatable promoter comprises the sequence of SEQ ID NO:2." Support for claim 39 appears in claim 15 as filed and in page 16, line 2. Support for claim 42 appears in claim 16 as filed, and on page 17, line 24 to page 18, line 2. Accordingly, claims having transcriptional repressor or activator proteins are adequately described in the specification. Claim 42 was also amended to more particularly point out the invention, deleting the "tetracycline repressor protein" and inserting "the transcription activation protein is a chimeric tetracycline repressor-Vp16 transcription activator polypeptide." These references to the supporting disclosure in the specification as filed provide proof of adequate written description for the presently pending claims, and thus the rejection should be withdrawn.

The Examiner contends that the specification does not provide adequate written description for tTA that is a tetracycline repressor. The terms "tetracycline repressor polypeptide" have been omitted from the claim language and thus the

rejection for lack of written description with respect to this contention should be withdrawn.

The Examiner further argues that the specification does not provide adequate written description for any repressible system other than the tet repressible system. In response, applicants respectfully direct the Examiner's attention to the extensive description of regulatable expression in the specification at page 12, line 19 through page 15, line 18, which includes both positive and negative regulatory elements, and provides examples such as the ecdysone, mifepristone, and estrogen systems. Attached are Shockett and Schatz (Nature Biotechnology 1997, 15:219-220, attached as Exhibit 1) and three papers cited therein, Shockett and Schatz (Proc. Natl. Acad. Sci. USA 1996, 93:5173-6, a review of diverse strategies for tetracycline-regulated inducible gene expression, attached as Exhibit 2), Feil et al. (Proc. Natl. Acad. Sci. USA 1996, 93:10887-90, reporting on a transgenic mouse with gene expression regulated by an estrogen analog, tamoxifen, attached as Exhibit 3); and Mattioni et al. (Methods Cell Biol. 1994, 43:335-352, steroid regulated fusion protein activators, attached as Exhibit 4).

The Nature Biotechnology review establishes that the state of the art with respect to regulatable promoters in transgenic animals was much farther advanced than the Examiner believes. Leaders in the field in 1997 recognized that "[i]n the past five years, several novel gene switches have been designed that appear to surpass earlier ones for use in transgenic mice" (page 219, column 1). The second

full paragraph on page 219 clearly establishes that inducing agents achieved the general features of increased specificity at non-toxic doses, without leaky expression. These gene switch transactivators can be expressed by constitutive, autoregulatory, tissue-specific, and development stage-specific promoters (page 219, column 1). Primary among them are the tet-regulated systems, which can be induced by addition or removal of tetracycline (page 219, paragraph bridging columns 1 and 2). As of 1997, "[u]se of the tTA systems, in all their forms, in conjunction with various reporter transgenes has recently been reviewed. [Shockett and Schatz, Proc. Natl. Acad. Sci. USA 1996, 93:5173]" (page 219, column 2) (emphasis added). The ecdysone, ecdysone/glucocorticoid hybrid (page 219, columns 2-3), and progesterone receptor (e.g., RU 486) based systems were also available (page 219, column 3). Various regulated expression systems used with the Cre-recombinase under control of cytokine inducible promoters or tamoxifen result in conditional knock-out animals (page 220, columns 1-2). "These basic systems share many positive features for inducible gene expression in animals: Gene activation over several orders of magnitude with low basal activity, and activators that have few side effects and no toxicity at functional doses in vivo" (id.). In short, as of the filing date of the patent application, the general ability to switch on gene expression in transgenic animals was state of the art.

In view of amendments to the specification and claims, and the arguments that the Applicant has made in response to each rejection, the Applicant

respectfully requests that the Examiner's rejections under §112, first paragraph for alleged lack of written description be withdrawn.

2. New Matter

The Examiner alleges that the phrase "chondrocyte tissue-specific promoter" is new matter. Applicant's respectfully submit that the phrase is not new matter. Applicants assert that support for the phrase appears in page 37, line 1, which discusses "tissue-specific expression conferred by the type II collagen promoter." At page 6, lines 4-5, the specification states that "... the recombinant MDE-encoding genes are selectively expressed in articular chondrocytes..."; likewise, at page 13, lines 3-4, "...MMP activity is selectively expressed in joint tissues, most preferably in articular chondrocytes". One of ordinary skill in the art would readily appreciate that the inventors had possession of a transgenic animal with chondrocyte tissue-specific expression of the transgene from this description. The Examiner's rejection fails to consider these express teachings in the specification. Accordingly, this rejection should be withdrawn.

3. Enablement

The Examiner has rejected claims 28-53 for alleged lack of enablement of the following:

(1) non-human transgenic animal;

(2) matrix degrading enzyme that degrades an extracellular matrix component;

(3) chondrocyte tissue-specific promoter.

With respect to point (1), the specification does provide guidance for the selection of non-human mammals in the production of transgenic animals other than the particular ones exemplified. Throughout the specification, reference is made to the production of transgenic animals. (*See* Specification at page 10, lines 11-19; page 21, lines 7-8). The specification clearly provides disclosure regarding a variety of methods employed by those of ordinary skill in the art both to generate transgenic animals and maintain such transgenics once made. (*See* Specification at page 23, lines 7 to page 26, line 8). For the reasons advanced in the Second Neuhold Declaration (attached as Exhibit 5), the specification enables claims to mammals. In particular, ". . . contrary to the examiner's assertions, as of 1996 creation of transgenic mammals required no more than ordinary technical efforts – indeed, technical efforts with shortcomings that are readily overcome" (Exhibit 5, paragraph 9). All of these techniques are set forth in the specification at pages 22-26.

The specification exemplifies a rat Type II collagen promoter (see the First Neuhold Declaration, attached as Exhibit 6). Mice and rats are well known to be closely related. The present invention exemplifies microinjection into fertilized

mammal embryos, which is the methodology used in production of all types transgenic animals and is extremely successful in this respect (see the Wall reference, attached as Exhibit 7).

It is well established that the Patent Office may not limit the claims to a narrow invention merely because specific examples of everything within the scope of a broad claim are not provided. *In re Anderson*, 176 USPQ 331, 333 (CCPA 1973). Indeed, a broader claim in a patent application can be enabled by disclosure of even a single embodiment, provided that undue experimentation is not required to practice the invention. *See Spectra-Physics, Inc. v. Coherent,* 827 F.2d 1524 (Fed. Cir. 1987). The proscription against undue experimentation is not a proscription against some experimentation. *See Amgen Inc. v. Chugai Pharmaceutical Co., Ltd.,* 927 F.2d 1200 (Fed. Cir. 1991). Indeed, even a complex and extended period of experimentation is not undue if the art engages in such experimentation and/or if the specification offers guidance or sufficient direction to one of ordinary skill in the art. *See* MPEP 214.01 and 2164.06. Moreover, it is not necessary under §112 to test in working examples all of the types of transgenic non-human mammals to be used in the practice of the invention. *See* MPEP 2164.02 and 2164.03.

Further, the constitutional purpose of the patent laws is to protect those that are the first to disclose an invention from others who seek to avoid infringement. The present inventor was the first to disclose functional constructs for the

generation of transgenic non-human mammals in which the transgene is selectively expressed in synovial chondrocytes and which provide for the generation of phenotypic indicators of pathological joint symptoms such as loss of proteoglycan, cleavage of type II collagen, gross observations of changes in joint function, histological evidence of (i) fibrillation and loss of articular cartilage and (ii) osteophyte formation, and combinations of said indicators. The specification discloses numerous methods for embryonic manipulations and other methods required in the generation of such transgenic non-human mammals. Applicants respectfully submit that failure to allow the broadest reasonable interpretation of the specification as recited in the present claims "would not serve the constitutional purpose of promoting progress in the useful arts." *See In re Groffe,* 542 F.2d 564, 567 (CCPA 1976); *see also* MPEP 2164.08.

The Examiner cites a reference detailing expression in other mammals as support for argument that the specification does not enable non-human mammals besides mouse. Applicants respectfully take issue with this citation on two grounds. First, because this reference establishes successful expression in another species, it supports the opposite conclusion: that the claimed transgenic animals are enabled. Second, this reference does not adequately support the rejection.

Mullins and Mullins, cited by the Examiner, reports that transgenic technology, including ES technology, is well established (page S37). Time and cost, issues irrelevant to enablement, limit the desirability of pronuclear injection in

larger mammals. No matter, as pointed out in the specification, ES technology is an alternative. In any event, the fact that pronuclear injection is less efficient, and therefore economically undesirable, fails to establish that it does not work. On the contrary, nothing in Mullins and Mullins supports such a conclusion as this paper reports on a number of successful non-murine transgenic animal models (see page S38).

The next substantive basis for rejection is the Examiner's contention that the specification does not teach a matrix degrading enzyme that degrades an extracellular matrix component. Enzymatically active matrix degrading enzymes that degrade extracellular matrix components are well known. *See* Specification at pages 2-3, 11-12; Second Neuhold Declaration (Exhibit 5, at paragraph 8). The specification teaches that certain MMPs are enzymatically active matrix metalloproteinases that cleave Type II collagen. As set forth in Table I on page 2 of the specification, the matrix metalloproteinases of this class include MMP-1 and MMP-13, both of which are regarded as important in osteoarthritis (specification, page 3, lines 4-10), and MMP-8. This subject matter is amply supported by the specification, as pointed out above.

With respect to the issue of constitutive enzymatic activity, applicants note that presently all the claims recite that the MMP is constitutively active. It is well known in the art that MMP's are produced as pro-enzymes that become active after removal of the pro-sequence (specification, page 11, line 22 to page 12, line 1). It

is also known that genetic engineering techniques, including mutations of the pro-sequence or modification to provide deletion of the pro region, can yield constitutively active MMP's (specification, page 12, lines 1-9). The Examiner has provided no evidence or reasonable ground for doubting the objective truth of these statements.

Finally, the Examiner has asserted that the specification does not enable any joint-specific promoter as claimed. Thus, the Examiner concludes that the claims should be limited to a Type II collagen promoter to regulate expression of the regulator polypeptide. Applicants cannot agree with this rejection, as it unduly limits the claims. The claims, after all, are directed to a particular type of transgenic animal, which successfully employs, but is not necessarily limited to, a Type II collagen tissue-specific promoter.

Support for the joint-specific promoter can be found in the specification at page 6, line 20. In addition, the specification clearly teaches that spatial control of MDE expression is achieved by the use of transcriptional promoters that direct transcription selectively in joint tissues. (*See* specification at page 15, lines 19-20). Such joint specific expression is clearly defined as that which produces expression in non-joint tissue of less than 10%, and preferably not at all. (*See* specification at page 15, lines 20-23). One source of such joint specific promoter sequences includes those derived from the collagen type II promoter. (*See* specification at page 16, lines 1-2). Finally, the specification teaches that such joint-specific

promoter sequences may comprise one or more copies of particular sequences or sub-sequences, and that these may be in direct or inverted orientation relative to each other or the sequence being regulated. (*See* specification at page 16, lines 2-6).

As set forth in the Second Neuhold Declaration (Exhibit 5, at paragraph 7), the specific promoter employed to achieve tissue specific expression does not make any difference, as one of ordinary skill in the art would readily appreciate. A number of issued patents that cover transgenic animals establish that tissue-specific expression is sufficiently enabled for expression of a transgene, because the actual tissue specific promoter is usually of no moment. Moreover, it is proper in a patent for a transgenic animal to claim the promoter by virtue of its tissue specificity rather than identity. *See* U.S. Patent Nos. 5,625,124 (claim 1: "gut epithelial cell specific promoter"); 5,880,327 (claim 1: "a mammary-gland specific promoter"); 5,917,123 (claim 1: "a cardiac-specific regulatory region"); 6,028,245 (claim 1: "a promoter that drives expression of the transgene in skin") (all attached as Exhibit 8). Again Applicant's request the Examiner to examine the invention as a whole. Taken as a whole, the Examiner offers no reasonable argument to question enablement of the claimed transgenic mammals when there is tissue specific expression of the MDE.

In view of the foregoing remarks, the Examiner's rejection for alleged lack of enablement under 35 U.S.C. §112, first paragraph, is overcome and should be

withdrawn.

35 U.S.C. §112, second paragraph

The Examiner has rejected claims 28-54 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite.

In particular, claims 28 and 41 are allegedly indefinite because the phrase "expression of the MDE . . . is repressed throughout embryonic, fetal, and early postnatal development, and activation of expression of the MDE results in a phenotypic change characteristic of osteoarthritis" is allegedly unclear. While applicants submit that the context of the claims makes it clear that the phrase is directly linked to the transgenic mammal, the claims have been amended to clarify this issue. The claims have also been amended to clarify the additional points cited by the Examiner. Thus, this basis for the rejection should be withdrawn.

The Examiner contends that the term "without proteolytic processing" in claim 30 is indefinite. While applicants submit that while the term "without proteolytic processing" is well understood in the art, in order to expedite allowance of the claims, the term "without proteolytic processing" has been deleted.

The Examiner contends that the metes and bounds of MMP-13 variants in claims 31 and 32 cannot be determined. In order to expedite allowance of the claims, the term "variant" has been deleted, as suggested by the Examiner.

The Examiner has objected to claim 35 as allegedly indefinite because "if the protein is a repressor, it cannot be an activator." The Examiner is correct in stating that if the protein is a repressor, it cannot be an activator. However, claim 35 refers to the repressor and activator in the alternative and does not state that the protein is both at once.

The Examiner has stated that claims 40, 45, 48 and 51 are allegedly indefinite because the genus of the Markush group uses the singular form while species within the Markush group use the plural form. These claims have been amended in accordance with the Examiner's suggestions and in order to expedite allowance of the claims.

The Examiner has rejected claims 44, 46, 49 and 52-54 as allegedly being indefinite because they allegedly fail to further limit the claim from which they depend because the mammal may already have MDE activated. Further the Examiner alleges that the distinction between "embryonic" and "fetal" is unclear and the metes and bound of "early" postnatal development cannot be determined. Applicant's have amended these claims to state that MDE expression is repressed until specific activation, and to replace "after embryonic, fetal, and early postnatal development" with "during adulthood." Accordingly, this basis for rejection is overcome and should be withdrawn.

The Examiner has also rejected claims 52-54 as allegedly indefinite because a change in phenotype does not indicate the composition counteracts osteoarthritis

as claimed. Applicant's have amended the claims to further clarify that any less extensive development in the nature or extent of the phenotypic change, or any increased length of time required for the phenotypic change to develop in the mammal that has been administered the composition relative to the control mammal indicates the potential of the composition to counteract the phenotypic characteristic of osteoarthritis. Support for this amendment can be found in the specification at page 20, line 13 to page 21, line 2 and in claims 25-27 as filed.

In view of the foregoing amendments and remarks, Applicants submit that these rejections are obviated and should be withdrawn.

CONCLUSION

Applicant's respectfully request entry of the foregoing amendments and remarks in the prosecution history of the application. It is respectfully requested that the application be reconsidered and allowance of pending claims is earnestly solicited.

If there are any other issues remaining which the Examiner believes could be resolved through either a Supplemental Response or an Examiner's Amendment, the Examiner is respectfully requested to contact the undersigned at the telephone number indicated below.

Respectfully submitted,

Date: April 30, 2002

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Attach #6

EXPRESS MAIL CERTIFICATE

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I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

Pat Campbell Lisa Love

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PATENT TRADEMARK OFFICE

Docket No: 0630/1D532US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Lisa Ann Neuhold and Loren Killar

Serial No.: 09/717,450 Art Unit: 1633

Confirmation No.: 5417

Filed: November 20, 2000 Examiner: Michael C. Wilson

For: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE

MARK-UP OF CLAIMS FOR AMENDMENT PURSUANT TO 37 C.F.R. §1.121

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

28. (Amended) A transgenic non-human mammal or progeny thereof whose [somatic and germline cells contain, in stably integrated form] genome comprises,

(a) a [first coding] nucleotide sequence encoding [an] a constitutively enzymatically active matrix degrading enzyme (MDE) that degrades an extracellular matrix component, wherein [expression of the first coding] the nucleotide sequence encoding the MDE is [under control of] operatively linked to a regulatable promoter [that is responsive to] comprising a transcriptional repressor or activator [polypeptide] protein binding sequence; and

(b) a [second coding] nucleotide sequence encoding [the] a transcriptional repressor or activator [polypeptide] protein, [wherein expression of the second coding sequence is] which nucleotide sequence encoding the transcriptional repressor or activator protein is operatively linked to [under control of] a chondrocyte tissue-specific promoter;

wherein expression of the MDE by chondrocytes is capable of being repressed in the mammal [throughout embryonic, fetal, early postnatal development] until adulthood, and [activation of expression of the] wherein MDE [results in a phenotypic change characteristic of osteoarthritis] is capable of being expressed in the mammal during adulthood to a level sufficient to cause degradation of an extracellular matrix component in the joints of the mammal.

30. (Amended) The transgenic mammal of claim 28, wherein the MDE is constitutively enzymatically active [without proteolytic processing].

31. (Amended) The transgenic mammal of claim 30, wherein the MDE is a constitutively enzymatically active MMP-13 [variant].

32. (Amended) The transgenic mammal of claim 31, wherein the MMP-13 [variant has a] comprises the sequence of SEQ ID NO:1 or SEQ ID NO:21.

35. (Amended) The transgenic mammal of claim 28, wherein the transcriptional repressor or activator [polypeptide] binding sequence is a repressor [polypeptide] binding sequence.

36. (Amended) The transgenic mammal of claim 35, wherein the repressor [polypeptide] binding sequence is a chimeric tetracycline repressor-Vp16 transcription activator polypeptide binding sequence.

37. (Amended) The transgenic mammal of claim 36, wherein the regulatable promoter [comprises a] is a tet07 [sequence] promoter.

38. (Amended) The transgenic mammal of claim 37, wherein the regulatable promoter comprises [a] the sequence [depicted in] of SEQ ID NO:2.

40. (Amended) The transgenic mammal of claim 28, wherein the

extracellular matrix component degradation results in a phenotypic change or changes [characteristic of osteoarthritis is] selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, [gross observations of changes] a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, [changes] a change in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

41. (Amended) A transgenic mouse or rat, or progeny thereof, whose [somatic and germline cells contain, in stably integrated form] genome comprises,

(a) [a first coding sequence] a nucleotide sequence encoding a constitutively enzymatically active human matrix metalloproteinase (MMP) that cleaves Type II collagen, wherein the nucleotide sequence encoding the metalloproteinase is operatively linked to a [expression of the first coding sequence is under control of a] tetracycline-regulatable promoter; and

(b) a nucleotide [second coding] sequence encoding a [tetracycline repressor polypeptide that] transcription activator protein, which transcription activator protein binds to the tetracycline-regulatable promoter, wherein expression of the nucleotide [second coding] sequence encoding the transcription activator protein is operatively linked to [under

control of] a chondrocyte tissue-specific promoter;

wherein expression of the MMP by chondrocytes is capable of being repressed in the mammal until adulthood [throughout embryonic, fetal, and early postnatal development], and [activation of expression of the MMP results in a phenotypic change characteristic of osteoarthritis in the transgenic mouse or rat] wherein the MMP is capable of being expressed in the mammal during adulthood to a level sufficient to cause Type II collagen degradation in the joints of the transgenic mouse or rat.

42. (Amended) The transgenic mouse or rat of claim 41, wherein the MMP is constitutively enzymatically active MMP-13, the tetracycline regulatable promoter is a tet07 promoter, the [tetracycline repressor polypeptide] transcription activation protein is a chimeric tetracycline repressor-Vp16 transcription activator [tTA] polypeptide, and the chondrocyte tissue-specific promoter [comprises sequences from] is a Type II collagen promoter.

43. (Amended) The transgenic mouse or rat of claim 42, wherein the Type II collagen degradation results in a phenotypic change or changes [characteristic of osteoarthritis is] selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, [gross observations of changes] a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, [changes] a change in growth plate morphology, fibrillation

and loss of articular cartilage, osteophyte formation, and combinations thereof.

44. (Amended) A method for producing degradation of an extracellular matrix component [a phenotypic change characteristic of osteoarthritis] in the joints of a transgenic mammal [of claim 28], which method comprises:

(a) repressing expression of MDE in a transgenic mammal of claim 28 until adulthood; and

(b) activating MDE expression in the transgenic mammal [after embryonic, fetal, and early postnatal development] after the mammal has reached adulthood such that the MDE degrades the extracellular matrix component in the joints of the transgenic mammal.

45. (Amended) The method according to claim 44, wherein the extracellular matrix component degradation results in a phenotypic change or changes [characteristic of osteoarthritis is] selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, [gross observations of changes] a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, [changes] a change in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

46. (Amended) A method for producing degradation of an extracellular matrix component [a phenotypic change characteristic of osteoarthritis] in the joints of a transgenic mammal [of claim 36], which method comprises:

(a) maintaining the transgenic mammal of claim 36 in the presence of [on] tetracycline or a tetracycline analog [during embryonic, fetal, and early postnatal development,] until adulthood; and

(b) activating the MDE expression by withholding the tetracycline or tetracycline analog from the mammal after the mammal has reached adulthood, such that the MDE degrades the extracellular matrix component in the joints of the transgenic mammal [after embryonic, fetal, and early postnatal development].

48. (Amended) The method according to claim 46, wherein the extracellular matrix component degradation results in a phenotypic change or changes [characteristic of osteoarthritis is] selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, [gross observations of changes] a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, [changes] a change in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

49. (Amended) A method for producing degradation of an extracellular

matrix component [a phenotypic change characteristic of osteoarthritis] in the joints of a transgenic mouse or rat [of claim 41], which method comprises:

(a) maintaining the transgenic mouse or rat of claim 41 in the presence of [on] tetracycline or a tetracycline analog [during embryonic, fetal, and early postnatal development,] until adulthood; and

(b) activating the [collagenase] MMP expression by withholding the tetracycline or tetracycline analog from the mouse or rat after the mouse or rat has reached adulthood, such that the MMP degrades the Type II collagen in the joints of the transgenic mouse or rat [after embryonic, fetal, and early postnatal development].

51. (Amended) The method according to claim 49, wherein the Type II collagen degradation results in a phenotypic change or changes [characteristic of osteoarthritis is] selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, [gross observations of changes] a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, [changes] a change in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

52. (Amended) A method for evaluating potential of a composition to counteract degradation of an extracellular matrix protein in joints of a mammal, which degradation results in a phenotypic change [characteristic of osteoarthritis,] selected

from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, a change in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof, which method comprises:

(a) administering the composition to the transgenic mammal of claim 28 in which a phenotypic change [characteristic of osteoarthritis] has been produced by activation of expression of the MDE [after embryonic, fetal, and early postnatal development] during adulthood of the transgenic mammal;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mammal to which the composition was administered relative to a control mammal in which expression of the MDE was activated without administering the composition,

wherein any [difference] less extensive development in the nature or extent of the phenotypic change, or any [difference in the] increased length of time required for the phenotypic change to develop[,] in the mammal that has been administered the composition relative to the control mammal indicates the potential of the composition to counteract the phenotypic change [characteristic of osteoarthritis].

53. (Amended) A method for evaluating potential of a composition to counteract degradation of an extracellular matrix protein in joints of a mammal, which degradation results in a phenotypic change [characteristic of osteoarthritis,] selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, a change in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof, which method comprises:

(a) administering the composition to the transgenic mammal of claim 36 in which a phenotypic change [characteristic of osteoarthritis] has been produced by activating expression of the MDE by withholding tetracycline or a tetracycline analog [after embryonic, fetal, and early postnatal development] during adulthood of the transgenic mammal;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mammal to which the composition was administered [relative] to a control mammal in which expression of the MDE was activated without administering the composition,

wherein any [difference] less extensive development in the nature or extent of the phenotypic change, or any [difference in the] increased length of time required for the phenotypic change to develop[,] in the mammal that has been administered the

composition relative to the control mammal indicates the potential of the composition to counteract the phenotypic change [characteristic of osteoarthritis].

54. (Amended) A method for evaluating potential of a composition to counteract degradation of an extracellular matrix protein in joints of a mammal, which degradation results in a phenotypic change [characteristic of osteoarthritis,] selected from the group consisting of loss of proteoglycan, cleavage of Type II collagen into a TC^A degradation product, a change in joint function, joint space narrowing, collagen degradation, destruction of cartilage, a change in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof, which method comprises:

(a) administering the composition to the transgenic mouse or rat of claim 41 in which a phenotypic change [characteristic of osteoarthritis] has been produced by activating expression of the MMP by withholding tetracycline or a tetracycline analog [after embryonic, fetal, and early postnatal development] during adulthood of the transgenic mouse;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mouse or rat to which the composition was administered [relative] to a control mouse or rat in which expression of the MMP was activated without administering the composition,

wherein any [difference] less extensive development in the nature or extent of the phenotypic change, or any [difference in the] increased length of time required for the phenotypic change to develop[,] in the mammal that has been administered the composition relative to the control mammal indicates the potential of the composition to counteract the phenotypic change [characteristic of osteoarthritis].

Respectfully submitted,

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RESEARCH NEWS

Switching on gene expression

Penny Shockett and David Schatz

Introducing extrinsic gene switches into transfected mammalian cells and transgenic mice has long been of interest to investigators seeking controlled expression of foreign genes that cannot be tolerated constitutively or during mouse development. Gene activation must be regulated precisely, quickly, reversibly, and be of sufficient magnitude for a given experimental situation. Gene expression systems initially developed for this purpose were problematic in mice because transgene expression was "leaky" or because nonspecific effects or toxicity arose from the inducing agents or treatments. In the past five years, several novel gene switches have been designed that appear to surpass earlier ones for use in transgenic mice. The latest example is reported by Wang and colleagues in this issue[1].

Recent systems employ inducing agents with increased specificity for target genes at nontoxic doses, and prevent gene activation in the uninduced state. They are regulated by the administration of drugs or hormones (or their analogs), and utilize modular transcriptional transactivators composed of natural or mutant drug/hormone ligand binding domains and intrinsic or extrinsic DNA-binding and transcriptional activation domains. These transactivators may be expressed by constitutive or autoregulatory ubiquitous promoters, or by tissue- and developmental stage-specific promoters that allow more precise spatiotemporal control. Ligand binding to transactivators either activates or prevents their interaction with specific DNA sequences upstream of the target genes, thereby suppressing or inducing expression. In all cases, transactivators are engineered for minimal homology to endogenous transactivators and minimal interaction with endogenous mammalian promoters.

Tetracycline (tet)-regulated systems, described initially by Gossen and Bujard[2], involve two versions of a transcriptional transactivator consisting of the DNA-binding domain of the tet repressor from *Escherichia coli*, fused to a herpes simplex virus (HSV) viral protein 16 (VP16) transcriptional activation domain. Target genes preceeded by tet operator sequences are activated by the binding of these transactivators. Binding by the transactivators to the operator is induced by addition (rtTA systems), or removal (tTA systems) of tet (or tet analogs)[3–5]. Use of tTA systems, in all their forms, in conjunction with various reporter transgenes has recently been reviewed[3]. The expression in mice of tTA under the control of tissue-specific promoters driving viral oncogene expression and subsequent cellular transformation is particularly noteworthy[6,7]. In addition, moderate *RAG* gene activity and lymphocyte development has been reconstituted in V(D)J recombination-activating gene (*RAG*) knockout mice expressing RAGs under the control of an autoregulatory version of tTA (ref 8; P. Shockett and D. Schatz, unpublished data).

Recent systems employ inducing agents, such as drugs or hormones (or their analogs), with increased specificity for target genes at nontoxic doses and prevent gene activation in the uninduced state.

Recently, No et al.[9] have described a system involving an insect hormone and its nuclear receptor. This system utilizes a heterodimeric ecdysone receptor complex consisting of a modified ecdysone receptor component (containing a DNA-binding domain and VP16 activation domain) and the mammalian homolog of its natural heterodimeric partner. In the presence of hormone, this receptor complex binds its response element in the promoters of target genes. Expression of the heterodimeric receptor, driven by the CD3 (T lymphocyte-specific) promoter, drives β-galactosidase mRNA expression at 48 hours only in mice given a synthetic analog of ecdysone. Ecdysone receptor/glucocorticoid receptor hybrid receptors and response elements that improve the specificity of the receptors and response elements for exogenous hormones and receptors, respectively, in transfected cells were also described, but these have not been tested in mice[9].

Now, Wang et al., using another hormone-based system, achieve liver-specific expression of human growth hormone (hGH) in transgenic mice[1]. In their system, the transactivator consists of a mutant human progesterone receptor ligand-binding domain fused to a yeast Gal4 DNA-binding domain and the VP16 transcriptional activation domain. Endogenous hormones cannot bind the mutant receptor domain of the transactivator. Transgenic mice were produced that express both the transactivator under the control of a liver-specific promoter, and a hGH target gene driven by upstream Gal4 consensus DNA-binding sequences. Liver-specific expression of hGH mRNA, serum hGH, and weight gain are detected only in mice given RU486, a progesterone analog used therapeutically in humans as a progesterone and glucocorticoid antagonist.

These basic systems share many positive features for inducible gene expression in animals: Gene activation over several orders of magnitude with low basal activity, and activators that have few side effects and no toxicity at functional doses in vivo. The rtTA system and the hormone receptors have in common the property that they are only activated in the presence of inducer[1,4,9]. Therefore, induction is relatively rapid and does not depend upon the half-life and subsequent clearance of the inducer.

In the Wang report, mice induced with a single injection of RU486 had serum hGH levels half-maximal at 8 hours and 3-orders of magnitude greater at 12 hours. The ability to control precisely the length of induction may be greater with the steroid hormones, given their short half-lives in vivo. Serum hGH was reduced approximately 80% by 2 days and completely by approximately 4 days after RU486 treatment. The lipophilic hormones might also more easily penetrate less accessible sites such as the brain. A novel feature of the progesterone receptor mice is the inclusion of transcriptional insulator sequences from the chicken β-globin locus, which are thought to minimize transgene integration effects. The fact that no side effects were observed from the inducers at effective doses makes them especially promising for human gene therapy applications.

The codevelopment of several syste

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involving distinct inducers and transactivators may prove extremely beneficial. Use of one system in conjunction with another may allow the differential regulation of more than one gene, or tighter regulation of the expression of one transactivator by another. Recent interest in creating conditional knockouts (or mutants) in vivo of genes that are embryonic lethal, has led to the development of systems employing inducible versions of Cre recombinase[10-12]. These systems involve either the expression of the recombinase under the control of cytokine-inducible promoters or the posttranslational activation of a modified recombinase protein by tamoxifen, an antiestrogen. In the future, it is likely that ligand-inducible transcriptional transactivators will be employed to drive expression in vivo of natural or ligand-inducible recombinase or other proteins.

1. Wang, Y., DeMayo, F.J., Tsai, S.Y., O'Malley, B.W. 1997. *Nature Biotechnology* 15:239–243.
2. Gossen, M. and Bujard, H. 1992. *Proc. Natl. Acad. Sci. USA.* 89:5547–5551.
3. Furth, P.A. et al. 1994. *Proc. Natl. Acad. Sci. USA.* 91:9302–9306.
4. Kistner, A., et al. 1996. *Proc. Natl Acad Sci USA* 93:10933–10938.
5. Shockett, P. and Schatz, D., 1996. *Proc. Natl Acad Sci. USA.* 93:5173–5176.
6. Efrat, S., Fusco-DeMane, D., Lemberg, H., Al Emran O., and Wang, X. 1995. *Proc. Natl Acad. Sci. USA* 92:3576–3580
7. Ewald, D. et al. 1996 Science 273:1384–1386
8. Shockett, P., Difilippantonio, M. Hellman N. and Schatz, D.G., 1995 *Proc. Natl Acad. Sci USA* 92:6522–6526.
9. No, D., Yao, T-P. and Evans, R.M., 1996 *Proc Natl Acad. Sci. USA.* 93:3346–3351
10. Kühn, R., Schwenk, F., Aguet, M. and Rajewsky, K., 1995. *Science* 269:1427–1429
11. Zhang, Y., et al., 1996. *Nuc. Acids Res.* 24:543–548
12. Feil, R., et al., 1996. *Proc. Natl. Acad Sci. USA* 93:10887–10890.

High-tech herbal medicine: Plant-based vaccines

Charles J. Arntzen

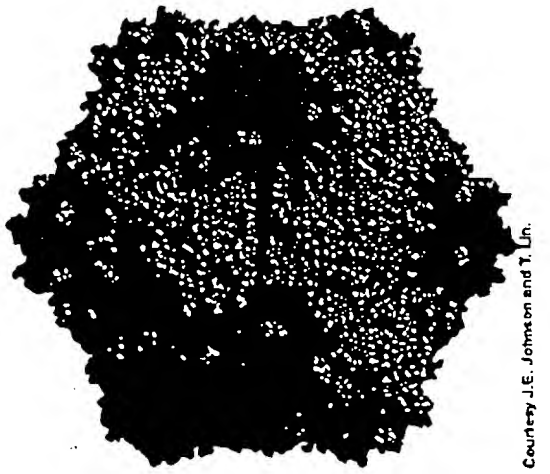
The long-held tenet "an apple a day keeps the doctor away" is closer to becoming scientific reality. Plants, which traditionally served as sources of food, fuel, and fiber, are now being engineered as novel biomanufacturing systems, with particular attention focused on the creation of plants that produce proteins of potential pharmaceutical value[1,2] or subunit vaccines[3]. On p. 248, Dalsgaard and colleagues[4] report another major step forward—the demonstration of a plant-derived subunit vaccine that protects animals from disease.

Traditional vaccines trigger an immune response in the recipient by injection or oral delivery of attenuated infectious agents that are incapable of causing disease (except in immunocompromised hosts or in other special circumstances). Over the past 15 years, a new vaccination strategy has emerged as the tools of molecular biology have identified the immunogenic proteins of the infectious agent that are responsible for the protective immune response. When the genes encoding these proteins are expressed in a heterologous system, and the resultant immunogens are isolated and utilized, they provide a subunit vaccine (i.e., the immunogenic subunit of the infectious agent).

Subunit vaccines offer a major advantage in that they are incapable of inducing disease—even in immunocompromised individuals—because they are free of the infectious agent. The design of subunit vaccines has become more sophisticated with increased understanding of the mechanism(s) for antibody binding to a particular part of an immunogen, a process that occurs at the antigenic determinant or epitope. Linear immunogenic epitopes have been defined through interactive immunology and protein engineering studies. These epitopes are linear sequences of the original immunogen that can elicit the desired host immune response when delivered as small protein fragments or as components of a fusion protein.

In their report, Dalsgaard and colleagues have fused the coding sequence for a linear epitope from an infectious animal virus (the parvovirus group) into the gene encoding the coat protein of a pathogenic plant virus. The entire viral genome sequence, including the component coding for the fusion protein, was then introduced into plants and shown capable of infecting and replicating in intact plants. From the plants infected with this construct, viruses were isolated that had the "foreign" epitope displayed on the surface of the coat protein. By itself, this is a very important advance in basic understanding of virus assembly; it builds on elegant previous studies that include analyses of the X-ray crystal structure of the plant/animal virus chimera[3] (see Figure 1). However, the authors go even further by evaluating the performance of the novel virus as a vaccine that protects animals from disease.

Two major strategies for the production of subunit vaccines in plants have been devised[1]: Genetic transformation of the nuclear genome of plants using gene vectors[5]; and manipulation of the genome of plant pathogenic viruses[2,4]. The most fundamental difference between these approaches is that the former gives rise to plants that have the "permanent" capacity to produce the desired vaccine. In contrast, the viral engineering approach gives a transient expression system in which the plants produce the immunogenic protein or peptide only when the plant is infected by the engineered virus. The latter strategy has the advantage of causing plants to produce relatively large amounts of the desired immunogenic protein, whereas transgenic plants have the advantage of constant levels of production of the proteins without intervention. It is likely that both approaches will have useful practical value under different circumstances.



Courtesy J.E. Johnson and T. Lin.

Figure 1. Structure of cowpea mosaic virus. The capsid comprises 60 copies each of the S coat protein (blue) and the L coat protein (domain I, light green; domain II, dark green). The loop into which foreign epitopes are inserted is shown in yellow.

Transgenic plants containing recombinant immunogenic proteins have been created by introducing coding sequences from

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Commentary

Diverse strategies for tetracycline-regulated inducible gene expression

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The recent development of tetracycline (tet)-regulated transactivation systems for inducible gene expression has dramatically enhanced the tools available for the temporal and quantitative control of exogenous genes in mammalian cells and transgenic mice and plants. Such systems have applications in many areas of biology and medicine, including the study of gene regulation and function in developmental systems; the role and biochemistry of particular genes in various biological processes; and the safe, controlled, administration of gene therapy. These systems have two central components: transcriptional transactivators that interact specifically with bacterial cis regulatory elements and antibiotics that modulate the binding of the transactivators at low, nontoxic doses. The consequence is a substantial reduction of nonspecific pleiotropic effects observed with earlier systems. Here we summarize the current status of the use of tet-regulated transactivation systems for the control of gene expression, including the contribution by Hoffman *et al.* in this issue of the *Proceedings* (ref. 1).

The first tet-regulated gene expression system for use in mammalian cells, developed by Gossen and Bujard (2), involved constitutive expression of the tet transactivator protein (tTA) with the human cytomegalovirus (CMV) immediate early (IE) promoter/enhancer. tTA is a fusion protein composed of the tet repressor of *Escherichia coli* and the transcriptional activation domain of the VP16 protein of herpes simplex virus. In the absence of tet, the tet repressor portion of tTA mediates high affinity, specific binding to sequences from the tet resistance operator of Tn10 (*tetO*). In the presence of tet, however, a conformational change in tet repressor prevents tTA from binding to its operator (3). Genes to be regulated by tTA (e.g., luciferase) were placed under the control of a hybrid, inducible promoter (hereafter referred to as tetP) which consists of a human CMV IE minimal promoter preceded by seven copies of *tetO*. In this initial study, performed in HeLa cells stably expressing tTA, expression of luciferase was very low in the presence of ng/ml quantities of tet, and removal of tet resulted in as much as a 100,000-fold increase in luciferase levels. Luciferase levels could be varied by titrating the amount of tet in the growth media, and maximal, steady-state levels of activity were achieved in about 24 h. Somewhat surprisingly, tTA was undetectable in the HeLa cells by Western blotting (although it was detected with a sensitive gel mobility shift assay), an observation consistent with toxicity of the tTA protein. This was speculated to be a consequence of transcriptional squelching, in which tTA would act as a sink for the general transcriptional machinery of the cell, resulting in the death of cells expressing moderate to high levels of tTA. Several technical and practical reviews of this system and its advantages over other inducible expression systems have appeared recently (4–6).

This basic system, since its description, has been used extensively in tissue culture for the expression of a variety of different genes. HeLa cells stably expressing tTA have been used to study the consequences of tet-regulated, tetP-controlled, stable expression of a number of different proteins involved in various areas of cell biology. These proteins include some that regulate the dynamics of proteosome subunit assembly, a viral protein that inhibits peptide transport across the endoplasmic reticulum membrane, and a mutant dynamin protein whose overexpression has consequences for endocytic-coated vesicle formation (6–9). This system has also been used to identify the targets of a viral transcriptional transactivator, to examine the phenotype of cells overexpressing a tyrosine kinase that regulates c-*src*, and to determine the consequences of disregulated expression of various cell cycle regulators both in HeLa cells and rat fibroblasts (10–14).

The basic tTA system has also been used to produce transgenic mice reversibly expressing luciferase or β-galactosidase in a variety of fetal and adult tissues (15). Expression was consistently highest in thigh muscle and tongue and was heterogeneous within these tissues. It was speculated that this heterogeneity might be inherent in the CMV IE promoter used to drive tTA or the was result of heterogeneous patterns of transgene methylation. These mice were created by breeding mice expressing tTA to mice expressing the reporter transgenes.

Subsequently, Gossen *et al.* (16) described a modified system in which a reverse transactivator (rtTA, or rtTA-nls, which contains a nuclear localization signal at its 5′-end) was developed that binds *tetO* efficiently only in the presence of the tet derivatives doxycycline or anhydrotetracycline. Using the CMV IE promoter to drive stable expression of rtTA in HeLa cells, luciferase activity could be induced by 3 orders of magnitude in 20 h by the addition of the tet derivatives. The vectors encoding these transactivators also contain a *neo*[r] cassette for selection in mammalian cells. It was proposed that this system would be especially useful in situations where cells or individuals were to be kept in the repressed state for long periods of time and where long term exposure to tet or its derivatives was undesirable or inconvenient (e.g., in gene therapy or transgenic animals), and in situations where one desired rapid induction, which might otherwise be limited by the rate of disappearance of tet from the system.

In an attempt to activate higher levels of gene expression than those obtained with the basic system, and to prevent possible toxic effects of constitutive tTA expression, we placed tTA under the control of tetP, resulting in the autoactivation of tTA in the absence of tet and suppression of tTA expression in the presence of tet (17). This autoregulatory system appeared to have two important advantages when compared with a system constitutively expressing tTA: it yielded substantially higher levels of target gene expression, and the frequency of inducible clones obtained was higher. We could readily detect tTA on Western blots, and optimal levels of the RAG proteins were detected after 12 h of induction by Western blotting (ref. 17, and unpublished data). Transgenic mice produced by coinjection of the autoregulatory tTA and a tetP-driven luci-

[illegible]

Abbreviations: tet, tetracycline; tTA, tet transactivator protein; CMV, cytomegalovirus; IE, immediate early; LTR, long terminal repeat; SV40, simian virus 40; MMTV, mouse mammary tumor virus.

erase transgene expressed luciferase inducibly in a variety of tissues with highest levels in thymus and lung (17). Induced luciferase levels were 1–2 orders of magnitude higher than those reported with the constitutive transgenic system (15), but the levels in the uninduced state were also greater. We have not determined whether more efficient suppression would be achieved with a more potent tet derivative, tet pellet implants, or doses of tet greater than 170 μg/ml (15, 16, 18). Luciferase was expressed in fetal tissues and continued to be expressed in mice bred and maintained in the absence of tet to 3.5 months of age. We are currently analyzing the heterogeneity of gene expression in these mice and the potential for breeding them to mice harboring other tetP-driven transgenes for activation in trans. It is important to note, however, that while the autoregulatory system has yielded high level expression in cultured cells (with induced mRNAs easily detected by Northern blotting of total RNA), expression levels in transgenic animals are substantially lower. We estimated that in thymus, where the highest induced levels of luciferase were observed, that cells contained on average only 30 molecules of luciferase protein. It is likely that further refinements of the system will be required for homogeneous, high level expression in transgenic animals.

Although the systems described above have been used successfully in many cell lines and to some extent in transgenic animals, some possible obstacles must be considered when attempting to use these systems. Cautions have been raised regarding the general efficacy of the systems in all cell or tissue types (19, 20). Because of the heterogeneity in gene expression that has been observed in some cases, it is generally agreed that success in any given cell or tissue milieu might require alternative minimal promoters and careful choice of constitutive or tissue-specific promoters for transactivator expression (refs. 19, 21, and commentary, ref. 22). For example, in mice carrying *lacZ* reporter transgenes activated by tTA expressed from a mouse mammary tumor virus (MMTV)-LTR, relatively homogenous expression was observed in epithelial cells of the seminal vesicle and salivary gland, and in Leydig cells of the testis, but heterogeneous expression was observed in mammary epithelial cells and basal cells of the epidermis (22). Another possible problem comes from the random nature of gene integration. Integration site-specific effects (such as constitutive activity or repression), which are inherent when foreign DNA is stably introduced into a cell or the mouse germline, might be overcome by surrounding individual transcription units with matrix attachment regions, shown previously to insulate stably integrated vectors and transgenes from effects mediated by cis regulatory elements adjacent to their sites of integration (22–25).

While the experiments described above utilize transactivator driven by ubiquitously active promoters, tet-regulated gene expression also holds much promise for experiments that require tissue-specific expression. Several recent experiments have demonstrated tissue-specific expression of transactivator directed by tissue-specific promoters. Cardiac-specific expression of tTA and subsequently luciferase protein or Id1 mRNA has been achieved using the rat α myosin promoter in rats or mice, respectively (see below) (26, 27). Expression of SV40 large T antigen in pancreatic β cells in mice was achieved using the original tTA system modified such that tTA was driven by the rat insulin promoter (RIP) (28). In induced mice, β-cell tumors were evident by 5–6 months of age, and transformed cell lines were derived that, upon restoration of tet, stopped proliferating, and that, upon implantation, were able to reverse hyperglycemia in diabetic mice. Inducible expression of CIITA, a transcription factor that regulates major histocompatibility class II expression, has also been achieved in mouse pancreas in which the rat insulin promoter drives tTA expression (C-H. Chang and R. A. Flavell, personal communication).

In some instances, for example during gene therapy, there is a need for timed or pulsatile expression of a given target gene in a particular tissue. The temporal requirement for viral oncogene expression for cellular transformation in the submandibular gland has recently been investigated in mice producing tTA under MMTV-LTR control and SV40 large T antigen driven by tetP (L. Hennighausen, personal communication). The ability to silence large T antigen expression at desired time points uncovered a time dependence of oncogene expression for persistent cellular transformation and underscores the utility of the system for controlling the timing of gene activation.

The quantitative regulation of gene expression by modulation of tet levels has not been analyzed as comprehensively in mice as in cell lines. However, experiments involving the transfection of skeletal muscle or heart tissue by the direct injection of DNA have addressed this issue. Oral, tet dose-dependent suppression of luciferase activity driven by the basic two plasmid system has been achieved in injected mouse skeletal muscle with maximal expression after tet removal achieved by 48 h (29). Expression of luciferase driven by tTA expressed under the control of the cardiac-specific rat α myosin promoter injected into cardiac tissue in rats also exhibited tet dose dependence (26). Additionally, transgenic mice expressing the cardiac specific tTA showed a more rapid and greater induction of Id1 mRNA expression in the heart upon removal of tet from mice bred and maintained on sub optimal doses of tet (0.1 mg/ml) compared with mice bred and maintained on higher doses (1 mg/ml), demonstrating that control of the kinetics and level of activation might be achieved by modulating the suppressive dose of oral tet (27).

Most reported studies of mammalian cell lines and transgenic mice made using either the constitutive or autoregulatory tTA systems have introduced transactivator and target genes on separate plasmids or transgenes. In stable cell lines DNA is introduced by transfection of individual plasmids consecutively or by cotransfection. Transgenic mice have been derived by breeding mice expressing transactivator to mice carrying the target gene and also by coinjection of transactivator and target DNA. An elegant study of transgenic tobacco plants by Weinmann *et al.* (30) demonstrated the feasibility of placing the tTA and reporter genes in opposing orientations on a single vector. tTA expression was controlled by either a plant-specific virus promoter or structure-specific promoter and the β-glucuronidase (*gus*) reporter gene was controlled by a minimal promoter with upstream tet operator sites. Tight regulation of expression by tet allowed the measurement of *gus* mRNA and protein half-lives.

Streamlined single vector expression systems for mammalian cells have also recently been developed and provide advantages for certain applications. Baron *et al.* (31) constructed a series of plasmids that contain two minimal promoters in opposite orientations on either side of the heptamerized tet operon allowing the tet-regulated expression of two genes in stoichiometric amounts from a single vector. Vectors expressing luciferase and β-galactosidase were described, which were vectors that allow mixing and matching between other genes and luciferase or β-galactosidase. It was suggested that such a plasmid, if modified to contain two different minimal promoters, might allow two genes to be co-regulated with different efficiencies. Another plasmid has recently been described that combines tTA (driven by a CMV promoter plus the SV40 late promoter) and the luciferase gene driven in the opposite orientation by tetP (32). COS cells transiently transfected with this plasmid (which also includes a *neo*^{r} gene for selection in mammalian cells) expressed luciferase at levels comparable with, and with the same degree of leakiness as, those transfected with the initial two-plasmid system. Tra

genic mice made with this vector express luciferase inducibly and reversibly with highest levels seen in thigh and abdominal muscles.

In an alternative method for delivery of tet-regulated genes, Paulus *et al.* (33) have adapted the one-vector approach for use in a retrovirus. The virus vectors contain tTA driven by either the SV40 promoter or a glial cell-specific promoter and either one or two copies of a luciferase gene driven in the opposite direction by tetP (33). These vectors also provide a gene for puromycin resistance. Induced luciferase activity (1–2 orders of magnitude) in infected cells was detected with these vectors upon removal of tet, although activity was not strictly glial-specific with the glial-specific promoter. It was proposed that in the vectors harboring a single luciferase gene (positioned along with the tTA gene between the viral LTRs) antisense inhibition of basal (but not induced) luciferase gene expression occurred as a result of transcription from the 5′-LTR.

Hoffman *et al.* (1) have now developed a system that merges the one-vector retroviral approach with the autoregulatory tTA expression strategy. This vector encodes a bicistronic mRNA allowing expression of both β-galactosidase and tTA from tetP, with tTA translation being initiated at an internal ribosome entry site. The virus self-inactivates during replication by deleting critical transcriptional control elements from the 5′-LTR, which prevents LTR interference with tet-regulated elements. Infection of primary myoblasts with this vector in the absence of tet resulted within days in β-galactosidase-expressing cells with a frequency expected for the viral titer used. The expression of β-galactosidase allowed for successive fluorescence-activated cell sorting and the subsequent enrichment of a population of cells with low basal and highest induced activity. Upon analysis of these selected cell populations, maximal activation of gene expression (1–2 orders of magnitude) upon removal of tet occurred by 48 h and was resuppressed 50% by 8 h. The authors discuss the need to include an additional fluorescence-activated cell sorting-detectable marker in the vector for the use of this strategy with target genes whose products cannot themselves be detected by fluorescence-activated cell sorting analysis. A bicistronic expression cassette encoding a tet-responsive target gene and a downstream alkaline phosphatase gene has also been used to screen stably transfected cells for low basal and high induced activity (34). The delivery of tet-regulated transgenes by retroviral infection is promising for some cell lines or primary cells that are difficult to transfect and possibly for gene therapy. Additionally, this method can theoretically eliminate integration site-specific effects that are averaged in uncloned populations of cells but which can become prominent in selected cell lines or clones.

Finally, another interesting virus-based approach involves the testing of vectors derived from the autonomous parvovirus, LuIII, for their ability to deliver tet-regulated gene expression units in tissue culture (35). These viruses, unlike retroviruses, are non-integrating, a desirable feature for those contemplating gene therapy strategies involving short-term delivery of a cytokine or toxin to certain cell types (for example in targeting suicide genes for cellular ablation in cancer therapy). These viruses have no pathological effects in humans, and they have a related mouse counterpart. In NB324K cells, transiently transfected with a vector driving tTA expression from the CMV IE promoter, expression of a tetP-driven luciferase gene introduced on a LuIII-derived vector was up to 200-fold higher in the absence of tet, compared with in the presence of tet. Titration of tet levels resulted in a dose-dependent increase in luciferase activity. These vectors will potentially be useful in situations where long-term persistence of a tet-regulated transduced gene is undesirable.

These early tet-regulated transactivation systems have already proven useful for addressing several basic experimental questions. Biologists and clinicians are actively working to apply these systems to a broader array of biological problems (e.g., the creation of conditional knockout mice using the Cre-loxP recombination system of bacteriophage P1) (36–38). With the continued cooperation between the many labs using the existing systems and the ongoing development of novel vectors, it seems likely that tet-regulated expression systems will play an important role in biological research and perhaps clinical medicine in the future.

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1. Hoffman, A., Nolan, G. P. & Blau, H. M. (1996) *Proc. Natl. Acad. Sci. USA* 93, 5185–5190.
2. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5547–5551.
3. Hinrichs, W., Kisker, C., Düvel, M., Müller, A., Tovar, K., Hillen, W. & Saenger, W. (1994) *Science* 264, 418–420.
4. Gossen, M., Bonin, A. L. & Bujard, H. (1993) *Trends Biochem. Sci.* 18, 471–475.
5. Barinaga, M. (1994) *Science* 265, 26–28.
6. Damke, H., Gossen, M., Freundlieb, S., Bujard, H. & Schmid, S. L. (1995) *Methods Enzymol.* 257, 209–220.
7. Früh, K., Gossen, M., Wang, K., Bujard, H., Peterson, P. A. & Yang, Y. (1994) *EMBO J.* 13, 3236–3244.
8. Früh, K., Ahn, K., Djaballah, H., Sempé, P., Van Endert, P. M., Tampé, R., Peterson, P. A. & Yang, Y. (1995) *Nature (London)* 375, 415–418.
9. Damke, H., Baba, T., Warnock, D. E. & Schmid, S. L. (1994) *J. Cell. Biol.* 127, 915–934.
10. Cayrol, C. & Flemington, E. K. (1995) *J. Virol.* 69, 4206–4212.
11. Bergman, M., Joukov, V., Virtanen, I. & Alitalo, K. (1995) *Mol. Cell. Biol.* 15, 711–722.
12. Resnitzky, D., Gossen, M., Bujard, H. & Reed, S. I. (1994) *Mol. Cell. Biol.* 14, 1669–1679.
13. Wimmel, A., Lucibello, F. C., Sewing, A., Adolph, S. & Müller, R. (1994) *Oncogene* 9, 995–997.
14. Shan, B. & Lee, W.-H. (1994) *Mol. Cell. Biol.* 14, 8166–8173.
15. Furth, P. A., St. Onge, L., Böger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H. & Hennighausen, L. (1994) *Proc. Natl. Acad. Sci. USA* 91, 9302–9306.
16. Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W. & Bujard, H. (1995) *Science* 268, 1766–1769.
17. Shockett, P., Difilippantonio, M., Hellman, N. & Schatz, D. G. (1995) *Proc. Natl. Acad. Sci. USA* 92, 6522–6526.
18. Gossen, M. & Bujard, H. (1993) *Nucleic Acids Res.* 21, 4411–4412.
19. Ackland-Berglund, C. E. & Lieb, D. A. (1995) *BioTechniques* 18, 196–200.
20. Howe, J. R., Skryabin, B. V., Belcher, S. M., Zerillo, C. A. & Schmauss, C. (1995) *J. Biol. Chem.* 270, 14168–14174.
21. Gossen, M. & Bujard, H. (1995) *BioTechniques* 19, 213–216.
22. Hennighausen, L., Wall, R. J., Tillmann, U., Li, M. & Furth, P. A. (1995) *J. Cell. Biochem.* 59, 463–472.
23. Stief, A., Winter, D. M., Strätling, W. H. & Sippel, A. E. (1989) *Nature (London)* 341, 343–345.
24. Phi-Van, L., von Kries, J. P., Ostertag, W. & Strätling, W. H (1990) *Mol. Cell. Biol.* 10, 2302–2307.
25. McKnight, R. A., Shamay, A., Sankaran, L., Wall, R. J. & Hennighausen, L. (1992) *Proc. Natl. Acad. Sci. USA* 89, 6943–6947.
26. Fishman, G. I., Kaplan, M. L. & Buttrick, P. M. (1994) *J. Clin. Invest.* 93, 1864–1868.
27. Passman, R. S. & Fishman, G. I. (1994) *J. Clin. Invest.* 94, 2421–2425.
28. Efrat, S., Fusco-DeMane, D., Lemberg, H., Al Emran, O. & Wang, X. (1995) *Proc. Natl. Acad. Sci. USA* 92, 3576–3580.
29. Dhawan, J., Rando, T. A., Elson, S. L., Bujard, H. & Blau, H. M. (1995) *Somatic Cell Mol. Genet.* 21, 233–240.
30. Weinmann, P., Gossen, M., Hillen, W., Bujard, H. & Gatz, C. (1994) *Plant J.* 5, 559–569.
31. Baron, U., Freundlieb, S., Gossen, M. & Bujard, H. (1995) *Nucleic Acids Res.* 23, 3605–3606.

32. Schultze, N., Burki, Y., Lang, Y., Certa, U. & Bluethmann, H. (1996) *Nat. Biotechnol.* **14**, 499–503.
33. Paulus, W., Baur, I., Boyce, F. M., Breakefield, X. O. & Reeves, S. A. (1996) *J. Virol.* **70**, 62–67.
34. Kirchoff, S., Köster, M., Wirth, M., Schaper, F., Gossen, M., Bujard, H., & Hauser, H. (1995) *Trends Genet.* **11**, 219–220.
35. Maxwell, I. H., Spitzer, A. L., Long, C. J. & Maxwell, F. (1996) *Gene Ther.* **3**, 28–36.
36. Lasco, M., Sauer, B., B. Mosinger, J., Lee, E. J., Manning, R. W Yu, S.-H., Mulder, K. L. & Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6232–6236.
37. Orban, P. C., Chui, D. & Marth, J. D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6861–6865.
38. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. & Rajewsk K. (1994) *Science* **265**, 103–106.

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Genetics

Ligand-activated site-specific recombination in mice

(inducible gene targeting/Cre recombinase/estrogen receptor/tamoxifen/somatic mutations)

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ABSTRACT Current mouse gene targeting technology is unable to introduce somatic mutations at a chosen time and or in a given tissue. We report here that conditional site-specific recombination can be achieved in mice using a new version of the Cre/*lox* system. The Cre recombinase has been fused to a mutated ligand-binding domain of the human estrogen receptor (ER) resulting in a tamoxifen-dependent Cre recombinase, Cre-ER^T, which is activated by tamoxifen, but not by estradiol. Transgenic mice were generated expressing Cre-ER^T under the control of a cytomegalovirus promoter. We show that excision of a chromosomally integrated gene flanked by *loxP* sites can be induced by administration of tamoxifen to these transgenic mice, whereas no excision could be detected in untreated animals. This conditional site-specific recombination system should allow the analysis of knockout phenotypes that cannot be addressed by conventional gene targeting.

The study of the genetic control of mammalian development and physiology has been revolutionized by the ability to inactivate (knockout) specific genes by homologous recombination in the mouse (1). However, using current gene targeting technology, interpretations of knockout phenotypes are often limited by several factors. First, the presence of a selection marker may influence the phenotype of the mutation (2, 3). Second, artefacts can arise due to the lack of a gene product for the whole lifetime of the animal. Third, the inactivation of a gene may result in intra-utero lethality, precluding analysis of the possible function(s) of the gene at later stages of development and/or post-natally. A conditional gene targeting method based on the inducible activity of an engineered DNA recombinase could overcome these limitations by allowing the removal of the selection cassette and the timed and tissue-specific inactivation of target genes at will during development and in the adult mouse (4). Furthermore, such an inducible system could help in certain cases to distinguish between anomalies related to a mixed genetic background and those due to mutation of the targeted gene.

The bacteriophage P1 Cre recombinase efficiently excises DNA flanked by two directly repeated *loxP* recognition sites in mammalian cells (5, 6). We have previously reported that fusion of the ligand-binding domain (LBD) of the estrogen receptor (ER) to the Cre recombinase generates a chimeric recombinase whose activity in cultured cells is dependent on the presence of an estrogen (estradiol) or an anti-estrogen (tamoxifen) (7). To achieve conditional gene targeting in mice, where endogenous estradiol is present, we have subsequently fused Cre to a mutated LBD of the human ER (Gly 521 → Arg, G521R) resulting in the chimeric protein Cre-ER^T. Indeed, the corresponding mouse ER LBD mutant (G525R) does not bind 17β-estradiol (E_2), whereas it binds the synthetic ligands tamoxifen and 4-hydroxytamoxifen (OHT) (8). We report here that Cre-ER^T is a functional tamoxifen-dependent recombinase in cultured cells and in transgenic mice.

MATERIALS AND METHODS

Construction of Plasmids and Generation of Transgenic Mice. pCMVCre-ER^T was constructed by cloning the 2-kb *Eco*RI fragment isolated from pCre-ER^T into the *Eco*RI site of the expression vector pMGSV1. pCre-ER^T was obtained by first replacing the 252-bp *Hin*dIII–*Bgl*II fragment of pCre-ER (7) with the corresponding fragment isolated from the expression vector HEG0 (9), coding for the human ER containing a glycine at amino acid 400. The amino acid corresponding to glycine 521 of the human ER was then mutated to an arginine by site-directed mutagenesis using the oligonucleotide 5′-CAC-ATGAGTAACAAAAGAATGGAGCATCTGTAC-3′. To obtain pMGSV1, a 600-bp *Xba*I–*Hin*dIII restriction fragment containing the enhancer/promoter region of the major IE gene of the human cytomegalovirus (CMV), isolated from pCMVcat (10) was first cloned into BSM− (Vector Laboratories cloning systems) digested with *Xba*I and *Hin*dIII, resulting in BSM–CMV. The simian virus 40 promoter region of pSG1 (11) was then replaced with the CMV promoter by cloning the 600-bp *Sac*I–*Hin*dIII restriction fragment isolated from BSM–CMV into pSG1 digested with *Sac*I and *Bam*HI, after filling in the *Hin*dIII and *Bam*HI restriction sites with T4 polymerase. The 4.6-kb *Pvu*II DNA fragment of pCMVCre-ER^T was injected into (C57BL/6 × SJL) F_1 zygotes at a concentration of 4 ng/ml to generate transgenic mice according to established procedures (12).

PCR Conditions. PCR amplification was carried out in a buffer containing 10 mM Tris·HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM of each primer, and 2 units *Taq* polymerase using 1 μg of genomic DNA as template. After 35 cycles (30 sec at 94°C, 30 sec at 55°C) the products were analyzed on ethidium bromide-stained 2.5% agarose gels.

Analysis of F9 Cells. Mouse F9 embryonal carcinoma cells carrying a floxed *tkneo* marker integrated into exon 4 of one retinoid X receptor α (RXRα) allele [RXRα$^{-/(LNL)}$, (13)] were transiently transfected with pCre-ER (7) or pCre-ER^T. After a 24-h incubation period, cells were grown in the presence of vehicle (ethanol) alone, 100 nM E_2, or 1 μM OHT for 48 h. PCR amplification of a 175-bp fragment specific for the excised allele was carried out using 1 μg of genomic DNA as template. The 5′ and 3′ primers were 5′-GGCAAACAC-TATGG-3′ and 5′-TTGCGTACTGTCCTCTT-3′, respectively.

Genotyping of Mice. The Cre-ER^T transgene and the RXRα$^{\Delta AF2(LNL)}$ target allele were detected in mouse tail DNA

Abbreviations: CMV, cytomegalovirus; Cre-ER^T, fusion protein between the Cre recombinase and a mutated ligand-binding domain of the human estrogen receptor (G521R); ER, estrogen receptor; LBD, ligand-binding domain; OHT, 4-hydroxytamoxifen; RT, reverse transcriptase; RXRα, retinoid X receptor alpha; TKneo, thymidine kinase/neomycin-resistance fusion gene; wt, wild type; HPRT, hypoxanthine phosphoribosyltransferase.

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both by PCR and Southern hybridization. For PCR detection of the Cre-ER^T transgene, the 5′ primer for *cre* was 5′-ATCCGAAAAGAAAACGTTGA-3′ and the 3′ primer was 5′-ATCCAGGTTACGGATATAGT-3′. For Southern blot analysis, tail DNA (10 μg) was digested with *Eco*RI and a 0.7-kb *Bam*HI–*Xho*I *cre* fragment isolated from pCre-ER (7) was used as a probe. For detection of the RXRα$^{\Delta AF2(LNL)}$ allele, the 5′ and 3′ PCR primers for *tkneo* were 5′-GGTTCTCCGGCCGCTTGGGT-3′ and 5′-GAAGGCGATGCGCTGCGAAT-3′, respectively. For Southern blot analysis, tail DNA was digested with *Bam*HI and probed with a 0.7-kb *Sac*I fragment of the RXRα gene.

Detection of Cre-ER^T mRNA Synthesis in Mice. The level of Cre-ER^T mRNA was estimated by reverse transcriptase (RT)-PCR. Total RNA was isolated from mouse tissues by the LiCl urea method (14). cDNA was synthesized for 20 min at 50°C using 50 units of Moloney murine leukemia virus RT and 1 μg of RNA and was then amplified by 35 cycles of PCR using primers 1 (5′-TTGACCTCCATAGAAGACAC-3′) and 2 (5′-GGCGATCCCTGAACATGTCC-3′), resulting in a 254-bp fragment of the Cre-ER^T cDNA. As an internal control, a 177-bp cDNA fragment of the hypoxanthine phosphoribosyltransferase (HPRT) mRNA was co-amplified in the same reaction using the 5′ and 3′ primers 5′-GTAATGATCAGTCAACGGGGGAC-3′ and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′, respectively.

Detection of Cre-ER^T-Mediated DNA Excision in Mice. PCR and Southern hybridization was used to analyze Cre-ER^T-mediated excision of the floxed *tkneo* marker from the RXRα$^{\Delta AF2(LNL)}$ target allele. PCR primers were primer 3 (5′-CAAGGAGCCTCCTTTCTCTA-3′) and primer 4 (5′-CCTGCTCTACCTGGTGACTT-3′). These primers amplify a 156-bp fragment of the RXRα wild type (wt) allele and a 190-bp fragment of the RXRα$^{\Delta AF2(L)}$ allele. Southern blot analysis was performed using a 2.0-kb *Kpn*I–*Bam*HI fragment or a 1.6-kb *Kpn*I fragment of the RXRα gene as hybridization probes. Genomic DNA was digested with *Bam*HI or *Nco*I.

Quantitation of DNA Excision and mRNA Levels. Semi-quantitative PCR was used to estimate the relative level of Cre-ER^T-mediated gene excision and semi-quantitative RT-PCR was used to estimate Cre-ER^T mRNA expression. For each sample the appropriate cycle number for remaining within the exponential phase was determined by running different cycle numbers (27, 30, 33, and 35). The ratio between the intensity of the bands derived from the excised allele and from the wt allele, as well as the ratio between the intensity of the bands derived from the Cre-ER^T mRNA and from the HPRT mRNA, was determined by laser densitometry of Polaroid photographs of ethidium bromide-stained agarose gels after subtracting the gel background. Only amplification reactions for which these ratios remained constant for at least two consecutive cycle numbers were used for quantitation. The percentage of Cre-ER^T-mediated deletion in a given organ was calculated from the intensities of the bands derived from the RXRα$^{\Delta AF2(L)}$ and wt RXRα alleles using the formula RXRα$^{\Delta AF2(L)}$/RXRα × 100. To estimate relative levels of Cre-ER^T mRNA expression, the Cre-ER^T signal obtained in a given tissue was normalized by division of the signal obtained for the HPRT standard mRNA. The value calculated for the skin was taken as 100%.

RESULTS AND DISCUSSION

To express a tamoxifen-dependent Cre recombinase, we constructed an expression vector, pCre-ER^T, encoding the fusion protein Cre-ER^T consisting of Cre fused to a mutated LBD of the human ER (G521R). The functionality of Cre-ER^T was tested by transient transfection using a mouse F9 embryonal carcinoma "reporter" cell line, which carries a chromosomally integrated "floxed" *tkneo* gene, i.e., a *tkneo* gene that is flanked by two directly repeated *loxP* sites. In these cells, Cre-ER^T excised the *tkneo* gene in the presence of 1 μM of OHT, but not in the presence of 100 nM of E_2 or in the absence of ligand, whereas, as reported (7), Cre-ER excised the target gene both in the presence of E_2 and OHT (Fig. 1). While the present study was in progress, a similar conditional Cre-ER^T recombinase was described (15).

Transgenic mice expressing Cre-ER^T under the control of a CMV promoter were generated (Fig. 2*a*). Out of three transgenic mouse lines, one showed stable maintenance and mRNA expression of the Cre-ER^T transgene in the tail (data not shown) and was used in all subsequent experiments. Mice expressing Cre-ER^T appeared phenotypically normal.

To analyze the efficiency of DNA excision by Cre-ER^T, we crossed Cre-ER^T mice with mice harboring a floxed target gene. This "reporter" line contains one wt allele of the RXRα gene and one modified RXRα allele carrying a floxed *tkneo* selection marker integrated by homologous recombination into the intron located between exon 5 and exon 6 [RXRα$^{\Delta AF2(LNL)}$, Fig. 2*b*, B.M., P. Kastner, and P.C., unpublished work]. After Cre-mediated excision of the marker gene one *loxP* site remains at this locus [RXRα$^{\Delta AF2(L)}$]. The wt RXRα allele and the excised RXRα$^{\Delta AF2(L)}$ allele can be simultaneously detected by polymerase chain reaction (PCR) using one set of primers (Fig. 2*b*). The relative efficiency of excision was estimated by comparing the intensity of the band amplified from the deleted RXRα$^{\Delta AF2(L)}$ allele with that of the band amplified from the wt RXRα allele, which differs in sequence only by the absence of the *loxP* site.

Offspring generated by crossing Cre-ER^T and RXRα reporter mice, which harbored both the Cre-ER^T transgene and the RXRα$^{\Delta AF2(LNL)}$ allele were identified by genotyping of tail biopsies (data not shown). These mice were treated with OHT at the age of 4 weeks and analyzed for Cre-ER^T-mediated DNA excision (Fig. 3*a*). Excision of the floxed marker gene was undetectable in oil-treated control animals (Fig. 3*a Upper* and data not shown), whereas mice injected intraperitoneally (i.p.) with OHT reproducibly showed excision of the floxed target gene in all organs tested except in the thymus (Fig. 3*a Lower* and data not shown). Importantly, in the tail the deleted RXRα$^{\Delta AF2(L)}$ allele was absent before OHT administration to the animal, whereas its presence was detected following OHT treatment [Fig. 3*a Lower*, compare tail(a) with tail]. The excision pattern and the absence of recombination background in control animals was confirmed using different routes of ligand administration (i.p., subcutaneous, orally) and Southern

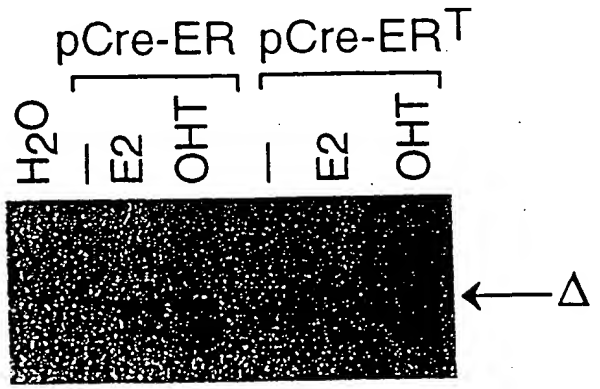

FIG. 1. Ligand-dependent activation of Cre-ER^T in mouse embryonal carcinoma cells. Excision of the *tkneo* gene in RXRα$^{\Delta AF2(LNL)}$ cells transfected with a control plasmid encoding an E_2-activated recombinase (pCre-ER) or with a plasmid encoding Cre-ER^T (pCre-ER^T) was analyzed by PCR. Cells were treated with vehicle (−), 1 nM E_2 or 1 μM OHT. A control reaction without DNA template is also shown. The position of the product amplified from the deleted allele (Δ) is indicated.

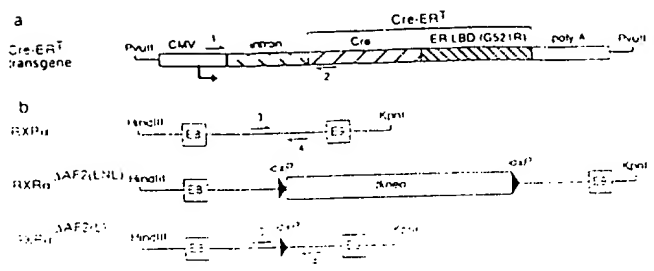

FIG. 2. (*a*) Structure of the Cre-ER^T transgene and strategy for detection of Cre-ER^T mRNA by RT-PCR. The DNA fragment used to generate transgenic mice contained the enhancer/promoter region of the major IE gene of the human CMV promoter, a rabbit β-globin intron, the Cre-ER^T consisting of the Cre recombinase gene fused to the cDNA coding for the G521R mutant of the human ER LBD, and a simian virus 40 polyadenylation signal [poly(A)]. The positions of the RNA startsite (arrow) and of the primers used for RT-PCR (primers 1 and 2) are indicated. (*b*) Genomic structure of the RXRα wt allele, the RXRα$^{\Delta AF2(LNL)}$ target allele, and the deleted RXRα$^{\Delta AF2(L)}$ allele, and PCR strategy (primers 3 and 4) to analyze Cre-ER^T-mediated excision of the floxed *tkneo* marker. Restriction sites are indicated.

blot analysis (data not shown). These results indicate that Cre-ER^T is a tightly regulated recombinase that displays undetectable activity in the absence of its cognate ligand and can be activated in mice by OHT treatment. We did not observe any deleterious effects of OHT treatment during this study, in agreement with reports indicating that tamoxifen has a very low acute toxicity and causes no severe anomalies in mice (16).

Cre-ER^T mRNA was detected in all organs analyzed except in the thymus (Figs. 3*b* and 4), suggesting that the protein is expressed in most tissues. Interestingly, the relative level of Cre-ER^T mRNA correlated well with the level of DNA excision in the various organs examined (Figs. 3 and 4). Excision was most efficient in tail, skin, kidney, and spleen where it occurred for 40–50% of the reporter allele. The level of excision was approximately 30% in the liver and stomach and less than 15% in other organs. In the tail, the excision remained at the same level (≈50%) after three or five injections of OHT (Fig. 4). Assuming that in the tail and possibly also in other tissues Cre-ER^T expression might be restricted to a subset of specific cells, the actual level of excision in these cells could in fact be higher. Note in this respect that CMV-driven transgene expression is known to vary considerably between different cell types in a given organ (17).

Cre-ER^T-mediated gene excision using the present Cre-ER^T expressing transgenic mouse line generates mosaic animals in which cells containing excised and nonexcised target DNA are mixed. Similar mosaics generated with mice homozygous for a targeted gene will allow the analysis of the function of this gene, provided its mutation results in a phenotype that does not depend on the inactivation of the gene in all cells of a given tissue. Furthermore, genetic mosaics can reveal several aspects of the mutant phenotype not necessarily apparent in knockout mutants (18), and also allow the analysis of mutations that result in lethality during embryogenesis or early post-natal development (19). Note that creating Cre-ER^T mouse lines expressing the conditional recombinase under cell/tissue-specific promoters will allow the selective mutation of a given gene at a given time in a given tissue. Note also that using an efficient inducible promoter system [e.g., one based on tetracycline inducibility (20)] that expresses the Cre-ER^T recombinase may be required to ensure that the recombinase levels are high enough to result in 100% excision of the targeted DNA.

Kuehn *et al.* (21) recently reported a method for conditional gene targeting in mice based on regulating the expression of a constitutively active Cre recombinase using an interferon-responsive promoter. The interferon-inducible system resulted in some tissues in higher rates of activated excision than those observed with the present Cre-ER^T mice, but was not as tightly controlled since background recombination was observed in interferon-untreated animals. We believe that further improvement of ligand-dependent Cre recombinase systems sim-

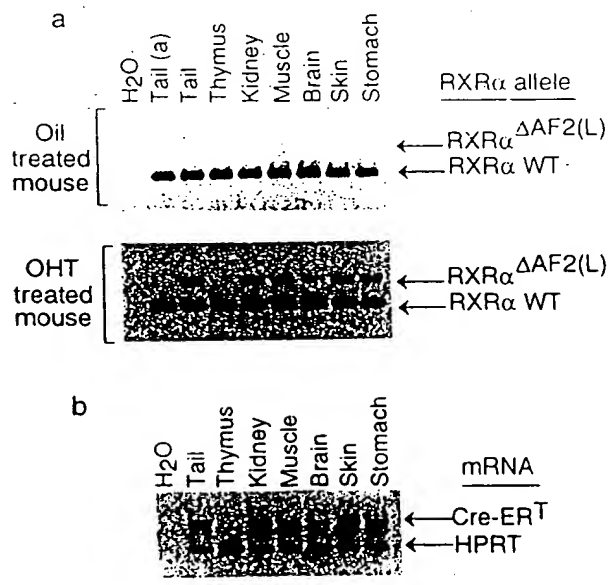

FIG. 3. (*a*) PCR detection of Cre-ER^T-mediated DNA excision in mice. Deletion of the floxed *tkneo* gene in various organs of untreated mice (*Upper*) and OHT-treated mice (*Lower*) was analyzed. PCR was also performed with tail DNA isolated before OHT administration [tail (a)]. Four-week-old Cre-ER^T/RXRα$^{\Delta AF2(LNL)}$ positive littermates were injected i.p. once per day with vehicle (oil) or with 1 mg OHT for 5 consecutive days. One day before the first injection DNA was prepared from tail biopsies of the animals. Two days after the last injection mice were killed and genomic DNA was isolated from various organs. The positions of the PCR products amplified from the RXRα allele and from the deleted RXRα$^{\Delta AF2(L)}$ allele are indicated. (*b*) RT-PCR analysis of Cre-ER^T mRNA expression. RNA was isolated from the same organs of an untreated littermate transgenic for Cre-ER^T, sacrificed on the same day (similar results were obtained when RNA was isolated from OHT-treated animals). PCR products corresponding to the Cre-ER^T mRNA and to the HPRT mRNA used as an internal control are indicated. Reactions run without DNA template are also shown. RT-PCR assays performed without RT did not result in any of the products (data not shown).

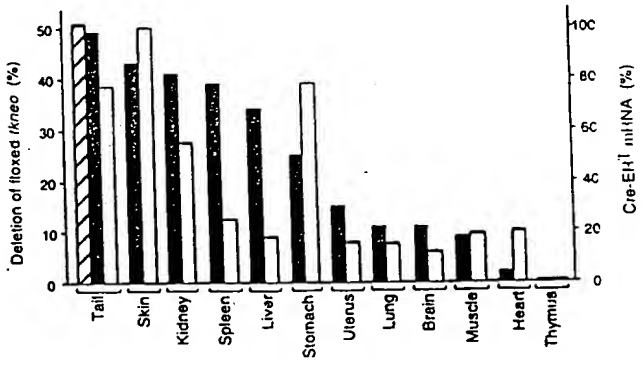

FIG. 4. Pattern of Cre-ER^T-mediated DNA excision and Cre-ER^T mRNA expression in various organs. The level of DNA excision after five i.p. injections of OHT in the indicated organs (shaded bars), the level of DNA excision in the tail 1 day after the third injection (hatched bar), and the corresponding levels of Cre-ER^T mRNA (open bars) are shown. Mice were treated as described in the legend to Fig. 3. No excision could be detected in untreated animals.

ilar to the present one will allow the manipulation of mouse genes to create somatic mutations in a spatio-temporally controlled manner.

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1. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51**, 503–512.
2. Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D. I. K., Enver, T., Ley, T. J. & Groudine, M. (1995) *Genes Dev.* **9**, 2203–2213.
3. Olson, E. N., Arnold, H.-H., Rigby, P. W. J. & Wold, B. J. (1996) *Cell* **85**, 1–4.
4. Marth, J. D. [illegible] **97**, 993–1002.
5. Sauer, B. & Henderson, N. (1990) *New Biol.* **2**, 441–449.
6. Sauer, B. & Henderson, N. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5166–5170.
7. Metzger, D., Clifford, J., Chiba, H. & Chambon, P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 6991–6995.
8. Danielian, P. S., White, R., Hoare, S. A., Fawell, S. E. & Parker, M. G. (1993) *Mol. Endocrinol.* **7**, 232–240.
9. Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I. & Chambon, P. (1989) *EMBO J.* **8**, 1981–1986.
10. Foeckling, M. K. & Hofstetter, H. (1986) *Gene* **45**, 101–105.
11. Green, S., Issemann, I. & Sheer, E. (1988) *Nucleic Acids Res.* **16**, 369.
12. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
13. Clifford, J., Chiba, H., Sobieszczuk, D., Metzger, D. & Chambon, P. (1996) *EMBO J.* **15**, 4142–4155.
14. Auffray, C. & Rougeon, F. (1980) *Eur. J. Biochem.* **107**, 303–314.
15. Zhang, Y., Riesterer, C., Ayrall, A.-M., Sablitzky, F., Littlewood, T. D. & Reth, M. (1996) *Nucleic Acids Res.* **24**, 543–548.
16. Furr, B. J. A. & Jordan, V. C. (1984) *Pharmacol. Ther.* **25**, 127–205.
17. Schmidt, E. V., Christoph, G., Zeller, R. & Leder, P. (1990) *Mol. Cell. Biol.* **10**, 4406–4411.
18. Nagy, A. & Rossant, J. (1996) *J. Clin. Invest.* **97**, 1360–1365.
19. Kastner, P., Mark, M. & Chambon, P. (1995) *Cell* **83**, 859–869.
20. Gossen, M., Freundlich, S., Bender, G., Müller, G., Hillen, W. & Bujard, H. (1995) *Science* **268**, 1766–1769.
21. Kuehn, R., Schwenk, F., Aguet, M. & Rajewsky, K. (1995) *Science* **269**, 1427–1429.

CHAPTER 16

Regulation of Protein Activities by Fusion to Steroid Binding Domains

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I. Introduction

A. A Novel Approach to Constructing Conditionally Active Proteins

With few exceptions, cell biologists have relied on inducible expression systems when they wanted inducible protein activity. Not surprisingly, this solution is often associated with severe disadvantages such as a long lag phase for protein accumulation. Until a few years ago, only a few alternative techniques,

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for example microinjection and the use of temperature-sensitive mutants, provided a way to circumvent the difficulties inherent in inducible expression systems. Table I compares the features of some existing inducible systems.

In this chapter we will discuss a novel approach that involves the post-translational regulation of a constitutively expressed fusion protein (Picard *et al.*, 1988; Yamamoto *et al.*, 1988; reviewed by Picard, 1993). This approach overcomes many of the shortcomings of the other systems mentioned in Table I. The method is based on the fact that the hormone binding domain (HBD) of steroid receptors can be used as an autonomous regulatory cassette to subject many heterologous protein functions to hormonal control *in cis*. Fusion proteins are maintained in an inactive state in the absence of hormone and are rapidly activated by addition of the cognate hormone.

B. A Model for the Regulatory Mechanism

We previously hypothesized that the inactivation of functions within the heterologous moiety of a fusion protein by the unliganded HBD is mediated

Table I
Induction Systems for Studying Proteins *in Vivo*[a]

System/approach	Activation of endogenous genes	Lag	Basal level	Number of plasmids introduced	Further comments
Viral infection (vaccinia, VSV, SV40, adenovirus, etc.)		Yes	No	0–1	Often severe side effects on cellular metabolism; host range restricted
Transcriptional systems					
T7, T3 RNA polymerase	No	Yes	Yes	1–2	Needs vaccinia superinfection for efficient translation
MMTV (GRE)	Yes	Yes	Yes	1	Requires endogenous steroid receptor
Heat-shock promoter	Yes	Yes	Yes	1	Severe side effects on cells
Metal-regulated promoter	Yes	Yes	Yes	1	Heavy metals are toxic
GAL4.ER.VP16	No	Yes	Yes	2	
lac repressor	No	Yes	Yes	2	Inducer IPTG inefficient and expensive
Tet repressor	No	Yes	Yes	2	Repression in presence of tetracycline
Microinjection[b]	No	No	No	0	Need protein; biochemical analysis difficult
Temperature-sensitive mutants[b]	Yes	No	No	1	Rarely available
Fusion to steroid binding domain[b]	**No**	**No**	**No–yes**	**1**	**Five different regulators**

[a] Examples are restricted to vertebrate systems, mostly tissue culture cells. The comparison, however, also applies to other organisms.

[b] Post-transcriptional systems.

by a complex containing heat-shock protein 90 (HSP90) (Picard *et al.*, 1988; Yamamoto *et al.*, 1988; further discussed by Picard, 1993). HSP90, as well as several other proteins, is associated with the unliganded HBDs of all five vertebrate steroid receptors, that is, the glucocorticoid (GR), mineralocorticoid (MR), androgen (AR), progesterone (PR), and estrogen (ER) receptors (for review, see Pratt, 1990; Smith and Toft, 1993). Hormone binding results biochemically in the release of the HSP90 complex and genetically in protein activation (or derepression). The hormone-reversible protein inactivation function of the HBD may therefore work by a relatively unspecific mechanism involving steric hindrance by the HSP90 complex. This method should be applicable to any protein with at least one essential function that is sensitive to steric hindrance. Hence, this regulatory system should work in the cytosolic and nuclear compartments of any organism that provides the components of the HSP90 complex.

II. Advantages and Limits

A. Advantages

The advantages can be summarized easily. (1) HBDs can subject a wide variety of heterologous proteins to hormonal control. (2) Induction (activation) occurs with very rapid kinetics (seconds to minutes) and is easily reversible. (3) Intermediate levels of induction can be obtained at subsaturating concentrations of hormone. (4) Activation of endogenous steroid receptors can be avoided by using a distinct one of the five different available HBDs, each with a distinct ligand specificity. (5) Given a set of five regulatory domains, several proteins can be regulated independently in the same cell. (6) The system has been shown to work in vertebrate cells and in yeast, and is likely to function in cells of a wide range of other organisms including plants and insects (for discussion, see Picard, 1993).

1. Regulation of a Wide Variety of Functions

A long list (Table II) of regulatable heterologous proteins has accumulated since the first report in 1988 showing that the adenovirus protein E1A can be subjected to hormonal control by fusion to an HBD (Picard *et al.*, 1988). Table II shows that the approach is not restricted to transcription factors. Notably, the list includes the RNA-binding protein Rev and tyrosine and serine/threonine kinases such as Abl, Src, STE11, and Raf1. Considering the aforementioned model, one could expect that the HSP90 complex might block any functional domain that requires the access of macromolecular partners (DNA, proteins, RNA, polysaccharides). In contrast, the interaction with small molecules might not be regulatable. Indeed, preliminary findings with β-galactosidase, galactoki-

Table II
Current List of Regulatable Heterologous Proteins

Protein X	Regulated as [a]	Reference
E1A (adenovirus)	Transcription factor	Picard *et al.* (1988)
E1A	Oncoprotein	Spitkovsky *et al.* (1994)
c-Myc	Oncoprotein	Eilers *et al.* (1989)
v-Myb	Transcription factor	Burk and Klempnauer (1991)
c-Fos, v-Fos, FosB-L, FosB-S	Oncoprotein, transcription factor	Superti-Furga *et al.* (1991); Schuermann *et al.* (1993)
Jun	Transcription factor	H. Beug and M. Busslinger, personal communication.
GCN4	Transcription factor	Fankhauser *et al.* (1994)
C/EBP	Transcription factor	Umek *et al.* (1991)
v-Rel	Oncoprotein, transcription factor	Bochmelt *et al.* (1992)
GATA-1, -2, -3	Transcription factor, promoter of proliferation (GATA-2)	Briegel *et al.* (1993)
GAL4-VP16	Transcription factor in yeast and in tissue culture cells	Braselmann *et al.* (1993); Louvion *et al.* (1993)
GAL4	Transcription factor in yeast	J.-F. Louvion and D. Picard, unpublished data.
MyoD	Transcription factor	Hollenberg *et al.* (1993)
p53	Transcription factor, tumor suppressor	Roemer and Friedmann (1993)
E7 (HPV16)	Oncoprotein	J. M. Bishop, personal communication.
Rev (HIV)	Transactivation (RNA-binding protein)	Hope *et al.* (1990)
c-Abl	Oncoprotein, tyrosine kinase	Jackson *et al.* (1993)
Src	Tyrosine kinase	J. M. Bishop, personal communication.
erbB1	Tyrosine kinase	J. M. Bishop, personal communication.
STE11	Serine/threonine kinase in yeast	J.-F. Louvion and D. Picard, unpublished data.
Raf1	Oncoprotein, serine/threonine kinase	Samuels *et al.* (1993)

[a] Fusion proteins were assayed in vertebrate tissue culture cells unless indicated otherwise.

nase, dihydrofolate reductase, and URA3 show that HBDs cannot inactivate their enzymatic functions in the absence of hormone (D. Picard, unpublished results). The enzymatic activity of dihydrofolate reductase fused to the GR HBD is even slightly reduced on hormone addition (Israel and Kaufman, 1993; D. Picard, unpublished results).

It is generally sufficient to regulate only one essential function of a heterologous protein to establish overall hormonal control. For example, in the case of a transcription factor, its activity will be hormonally regulated irrespective of whether it is only the dimerization, the nuclear localization, the DNA binding, or the transcriptional regulatory function that is directly inactivated by the

HBD. Indeed, there are few cases where the precise activity that is inhibited has been determined (for discussion, see Picard, 1993).

2. Combinatorial Regulation with Several Signals

Experimentally, a hormone-reversible protein inactivation function has been demonstrated for the HBDs of GR (Picard *et al.*, 1988), ER (Eilers *et al.*, 1989), MR (Fankhauser *et al.*, 1994), and AR (F. Stewart, personal communication). Our model predicts that the HBD of PR should also work. Hence, in all likelihood, there are up to five different regulatory domains that can all be regulated independently and specifically with their cognate ligands. One can therefore avoid the activation of endogenous steroid receptors, which may be expressed in a particular vertebrate cell line, by choosing the appropriate regulatory domain. It should often be possible to regulate several fusion proteins, each with a different HBD, in the same cell.

Whether the ligand binding domains of other members of the nuclear receptor superfamily may be widely applicable for this type of regulation is currently unclear. The HBDs of the thyroid receptor (Hollenberg *et al.*, 1993) and the *Drosophila* ecdysone receptor (Christopherson *et al.*, 1992) have been reported to work for the regulation of fusion proteins. However, we have been unable to confirm this finding for the ecdysone receptor HBD using Fos as a test protein (M. Worek and D. Picard, unpublished results). Since these types of HBDs do not appear to form hormone-reversible stable complexes with HSP90, their regulatory potential may be limited to special cases and may depend on the interaction with other proteins such as the retinoic acid X receptor (RXR).

B. Limits

The following concerns will be discussed in this chapter: (1) Will the heterologous protein tolerate a fusion? (2) Does the fusion protein have qualitatively altered properties? (3) Is there a basal level of expression?

The approach can only work if the protein of interest tolerates fusion to the rather large (about 300 amino acids) regulatory domain. It is often difficult to predict whether N- or C-terminal or even internal additions are compatible with the activity of the heterologous moiety. The number of successful examples (see Table II) is indeed quite surprising, confirming that many proteins consist of fairly independent modules.

A priori it is difficult to exclude the possibility that the activity of a fusion protein may be different than that of the wild-type unfused protein. Remarkably, fusion proteins have largely been found to have full or only slightly reduced activity after hormone induction (see references in Table II). A major concern is the possibility that the fusion protein may have qualitatively different properties than the wild-type protein. This concern stems from the complication that

HBDs carry more than the inactivation function; they also contain hormone-dependent nuclear localization (Picard and Yamamoto, 1987; Guiochon-Mantel *et al.*, 1989; Ylikomi *et al.*, 1992), dimerization (Kumar and Chambon, 1988; Wrange *et al.*, 1989; Fawell *et al.*, 1990), and transactivation (denoted TAF-2) functions (reviewed by Gronemeyer, 1991). With one exception (see subsequent discussion), significant alterations of activity have not been reported to date and should be avoidable in most cases. Only the HBD of GR contains an autonomously active and hormone-dependent nuclear localization signal (Picard and Yamamoto, 1987; Picard *et al.*, 1990b). The dimerization activity of the HBD appears to be relatively weak, which is consistent with the notion that fusion proteins with Fos (Superti-Furga *et al.*, 1991) and Myc (Eilers *et al.*, 1989) were still able to heterodimerize with their partners Jun and Max, respectively. The transactivation function of the HBD (TAF-2) is a potential problem in fusions with transcription factors. The contribution of TAF-2 has been shown to be considerable with weak transactivators (Schuermann *et al.*, 1993). However, this problem could be avoided altogether by using specific TAF-2 mutants (Danielian *et al.*, 1992; Ince *et al.*, 1993; Wrenn and Katzenellenbogen, 1993). Note that HBD functions other than the inactivation function have also been exploited to regulate heterologous proteins. The nuclear localization and dimerization functions have been exploited for the regulation of Rev (Hope *et al.*, 1990,1992) and c-Abl (Jackson *et al.*, 1993), respectively.

Another common concern about inducible systems is the basal level of activity under uninduced conditions. Ultimately the basal level of activity of a fusion protein must be determined experimentally. As for any other inducible system, the basal level that can be tolerated will depend on the biological assay system. Note also that the position of the HBD relative to active sites in the fusion protein will determine whether an activity is inhibited. As previously summarized (Picard, 1993), often only a subset of all functions of a heterologous moiety is regulated. Despite considerable flexibility of the regulatory mechanism, the tightness of regulation must depend on the positioning of heterologous function(s) relative to the HBD or, more specifically, to components of the HBD–HSP90 complex. This issue will be further illustrated in our discussion of E1A fusion proteins.

III. Designing and Using Fusion Proteins

A. Choice of Hormone Binding Domain

The choice of HBD is primarily dictated by the biological system rather than by the heterologous protein itself, since the activation of endogenous steroid receptors is usually undesirable. Although the expression pattern of steroid receptors may not be known for a given cell line, the literature on the tissue- and cell-type-specific effects of steroid hormones is vast. GR is almost ubiquitously expressed, at least in mammals, whereas other steroid receptors are much more

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tissue specific; MR may display the narrowest distribution. Indeed, by choosing between the MR and ER HBDs, cross-talk with endogenous receptors should almost always be avoidable. Free choice applies to other organisms such as yeast, plants, and invertebrates because vertebrate steroids tend to have no or little effect on their physiology.

Although the GR HBD has been used extensively (Picard *et al.*, 1988; Eilers *et al.*, 1989; Hope *et al.*, 1990,1992; Superti-Furga *et al.*, 1991; Umek *et al.*, 1991; Hollenberg *et al.*, 1993; Jackson *et al.*, 1993; Louvion *et al.*, 1993), it should be reserved for special cases. Disadvantages of the GR HBD are (1) the potential cross-activation of endogenous GR and (2) a severe loss of hormone binding affinity by about two orders of magnitude when the HBD is isolated from the intact GR (Rusconi and Yamamoto, 1987). As a consequence, the addition of high concentrations of glucocorticoids may even cross-activate other steroid receptors such as the MR. Moreover, in yeast very high ligand concentrations are needed to induce GR (see, for example, Picard *et al.*, 1990a,c; Wright *et al.*, 1990; Garabedian and Yamamoto, 1992; Wright and Gustafsson, 1992); thus, it may even be difficult to achieve sufficient hormone concentrations for full activation of the GR HBD.

In contrast, mutant HBDs with only moderately reduced hormone binding affinity are extremely useful tools to avoid activation by steroids that may be present in the biological assay system. For example, it is often difficult to avoid partial activation of the wild-type ER HBD by estrogens in tissue culture medium. The point mutation Gly 400 to Val 400 in the human ER reduces affinity about 10-fold (Tora *et al.*, 1989), rendering the HBD less sensitive to estrogenic contaminants in medium. HBD mutants with altered specificities offer the additional advantage that endogenous receptors may not be activated or even repressed. For example, a C-terminal deletion mutant of the human PR has been described that, instead of being activated by progesterone, now responds to RU486, normally a PR antagonist (Vegeto *et al.*, 1992). Moreover, several point mutations in the ER HBD strongly reduce estrogen binding and receptor activation but allow the estrogen antagonist hydroxytamoxifen to act as an agonist (Danielian *et al.*, 1993; Ince *et al.*, 1993; Wrenn and Katzenellenbogen, 1993).

B. Designing Fusion Proteins and Their Expression Vectors

For maximal tightness of control, it is advisable to place the HBD relatively "close" to an essential function of the heterologous moiety despite all the apparent flexibility of the regulatory mechanism. Spacial proximity is almost certainly critical. Although the spatial arrangement usually remains undetermined, it may often be reasonably approximated by the primary structure. In principle, the HBD can be fused to the N terminus (Eilers *et al.*, 1989) or the C terminus (vast majority of examples) or inserted into the protein (Picard *et al.*, 1988; Braselmann *et al.*, 1993; Hollenberg *et al.*, 1993; Louvion *et al.*,

1993). Fusion of the HBD, a C-terminal domain in wild-type receptors, to the C terminus of another protein is obviously easier to achieve since it only requires one in-frame junction. An additional complication with N-terminal additions may be the possibility for translation initiation at an internal AUG, resulting in the partial or complete deletion of the regulatory HBD from a subset of molecules. This appears to explain the elevated basal level of ER-Myc fusion proteins compared with Myc-ER in the absence of hormone (Eilers *et al.*, 1989; M. Eilers, personal communication).

Vectors for constitutive or even inducible expression *in vivo* can be chosen according to the needs of a given biological assay system. Note that proper hormonal regulation cannot be expected to work in bacteria or cell-free systems. In the latter case, the conditions for hormonal regulation and assay of the heterologous activity (for example, by gel shift) are often incompatible.

C. Tissue Culture Media

To avoid the activation of fusion proteins by steroid "contaminants" present in culture medium the following precautions may be necessary.

1. Media without Phenol Red

Phenol red, added to the majority of mammalian cell culture media as a pH indicator, has been shown to mimic the effect of certain steroids (Berthois *et al.*, 1986; Picard and Yamamoto, 1987). Therefore, it is preferable to use media without phenol red. Such media are now commercially available.

2. Removal of Steroids from Serum by Charcoal Treatment

Serum, added as a supplement to the culture medium for many cell lines of mammalian and non-mammalian origin, is a source of steroid contaminants. These contaminants can be eliminated by charcoal treatment:

1. To 100 ml serum, add 2 g acid-washed activated charcoal (e.g., Sigma, St. Louis, MO).
2. Stir for 90 min at 4°C.
3. Remove the charcoal by passage through filter paper (e.g., pleated filter; Schleicher & Schuell, Keene, NH).
4. Sterilize the serum by filtration (0.22-μm filter).

Note that charcoal treatment of the serum may deplete other important components. Therefore, certain cell lines or cell types may not be able to grow in medium supplemented with charcoal-treated serum. As an alternative, a synthetic serum substitute could be tested [e.g., Nutridoma (Boehringer Mannheim, Indianapolis, IN) or NuSerum (GIBCOBRL, Grand Island, NY)]. These particular serum substitutes are completely steroid free. However, certain cells may

fail to grow with any of these medium supplements. In this case, they could be maintained in normal complete medium and only switched to the special medium for the last 24–48 hr of a short-term experiment (e.g., a transient transfection experiment).

D. Activation and Deinduction

The induction of fusion proteins is achieved by adding specific ligands directly to the medium (Table III). For deinduction hormone is removed by washing the cells several times with medium, phosphate-, or Tris-buffered saline prior to feeding them with fresh complete medium devoid of specific ligand. In long-term experiments, the medium is replaced again 24 hr later.

IV. Examples

Three representative examples will be discussed. (1) E1A: This adenovirus early protein can be regulated as a transcription factor or an oncoprotein, depending on the relative positioning of E1A domains and the HBD. (2) Abl: As a fusion protein, this nonreceptor tyrosine kinase has two opposite activities: it is an oncoprotein and a growth inhibitor in the presence and absence of hormone, respectively. (3) STE11: The regulation of this serine/threonine kinase of the yeast pheromone signaling pathway illustrates that fusion proteins can also be used in yeast.

Table III
Hormone Binding Domains and Their Cognate Ligands

HBD	Amino acid positions[a]	Ligand	Final concentration[b]
GR	500–540 to 793–795 (rat)	Dexamethasone	10 μM
MR	685 to 981 (rat)	Aldosterone	10 nM
ER			
Wild-type G400	282 to 576–595 (human)	17β-Estradiol	0.1 μM
Mutant V400	282 to 576–595 (human)	17β-Estradiol	0.1 μM
AR	~650 to 918 (human)	5α-Dihydrotestosterone	0.1 μM
PR	~670 to 933 (human)	Progesterone	0.1 μM

[a] For the GR and ER HBDs there is an experimentally determined flexibility with respect to N and C termini. Amino acid positions are characteristic for the species indicated in parentheses, but given the extensive evolutionary conservation the corresponding HBD sequences from other species can be utilized as well.

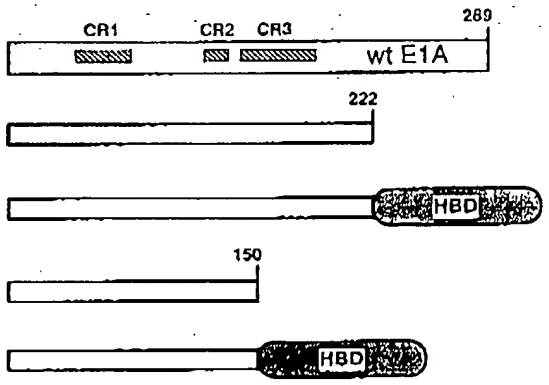
[b] Ligands and concentrations are appropriate for vertebrate tissue culture cells and should not yield cross-activation of other endogenous steroid receptors. For other organisms, both the type of ligand and the concentration may have to be adapted. In the yeast *Saccharomyces cerevisiae*, 10 μM deoxycorticosterone should be used with the GR and MR HBDs. Hormones can be stored as 1000-fold concentrated stock solutions in ethanol at −20°C.

A. E1A

E1A is an adenovirus early protein that can activate the transcription of several adenoviral and cellular genes, apparently by being tethered to DNA indirectly via a partner protein (reviewed by Nevins, 1990). E1A can also immortalize certain types of primary cells and can collaborate with other oncoproteins to transform fibroblast cells (Land *et al.*, 1983; Ruley, 1983; Shenk and Flint, 1991). With E1A as an example (Fig. 1), we will emphasize how fusion proteins are used in short-term ("transient") and long-term ("stable") gene transfer experiments and how different functions of a heterologous fusion protein can be differentially regulated by an HBD.

1. Transactivation

Conserved region 3 (CR3) of E1A is necessary and sufficient for transactivation (reviewed by Flint and Shenk, 1989). Therefore, the N-terminal 222 amino acids of E1A, which retain CR3, were fused to the GR HBD. Picard *et al.* (1988) demonstrated that such E1A–HBD fusion proteins enhance transcription of a reporter plasmid in a fully hormone-dependent fashion. Typically, expression plasmid for the effector protein and reporter plasmid are co-transfected into HeLa or CV1 cells by calcium phosphate co-precipitation. Cells are washed



Transactivation		Transformation	
- H	+ H	- H	+ H
yes	yes	yes	yes
yes	yes	yes	yes
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Fig. 1 Differential regulation of E1A functions by a steroid hormone binding domain (HBD). The transactivation and transformation functions of E1A require conserved regions CR3 and CR1–CR2, respectively. In fusion proteins these regions can be subjected to hormonal control by the HBD depending on the relative positioning of regulatory and functional domains (after Picard *et al.*, 1988; D. Picard, unpublished results). wt, Wild-type; transactivation, transcriptional stimulation of an E1A-reponsive reporter gene; transformation, collaboration with the *ras* oncogene in immortalizing and transforming primary fibroblast cells; H, steroid hormone (added as indicated in Table III).

18 hr after adding DNA, and glucocorticoid hormone is added with fresh medium for another 24 hr before harvesting the cells. In this case, hormone is present during accumulation of the fusion protein. Alternatively, hormone can be added once fusion protein levels reach a maximum since regulation is post-translational. Using this approach, it could very easily be confirmed, by adding cycloheximide during hormonal induction, that protein synthesis is not required for E1A action (D. Picard, unpublished results).

2. Hormone-Reversible Transformation

The collaboration of E1A with the *ras* oncogene in the transformation of fibroblasts, which requires CR1 and CR2, can be assayed by co-transfecting expression vectors for an E1A fusion protein and for Ras into primary rat embryo fibroblast cells. Within 2–3 wk, transformed cells will form foci. If they are also immortalized, they can be established as a permanent cell line.

The transformation function turns out to be constitutively active in fusion proteins retaining the first 222 amino acids, including CR3 (Fig. 1). In the case of the "long" E1A fusions, the transformation function is not regulated (D. Picard, unpublished results) whereas the transactivation function is (Picard *et al.*, 1988). However, even the former could be regulated by moving the HBD (from GR or ER) closer to CR1 and CR2 on the linear protein map (D. Picard, unpublished results; Fig. 1). This observation is consistent with the idea that physical proximity of the HBD may be important for tight regulation despite remarkable spatial flexibility.

Such a shortened E1A fusion protein induces foci in primary cells exclusively and reversibly, in the presence of steroid hormone. In fact, we have been able to derive a permanent cell line that grows only in the presence of steroid hormone and becomes arrested in G_1 after hormone removal (Spitkovsky *et al.*, 1994).

B. Abl

The product of the c-*abl* proto-oncogene is a member of the nonreceptor class of protein tyrosine kinases. As a consequence of genetic alterations such as viral transduction or chromosomal translocation events, c-*abl* can acquire the capacity to induce malignant transformation (reviewed by Rosenberg and Witte, 1988; Daley and Ben-Neriah, 1991). In addition, several transforming variants of c-*abl* have been constructed by *in vitro* mutagenesis (Jackson and Baltimore, 1989). Oncogenic transformation is reflected, for example, by altered morphology and growth properties and always correlates with increased Abl tyrosine kinase activity. To facilitate the investigation of the transformation process, regulatable Abl derivatives have been constructed by fusion to the HBD of the ER (Fig. 2A; Jackson *et al.*, 1993). Here we describe the protocols used to generate and to study cell lines containing these fusion proteins.

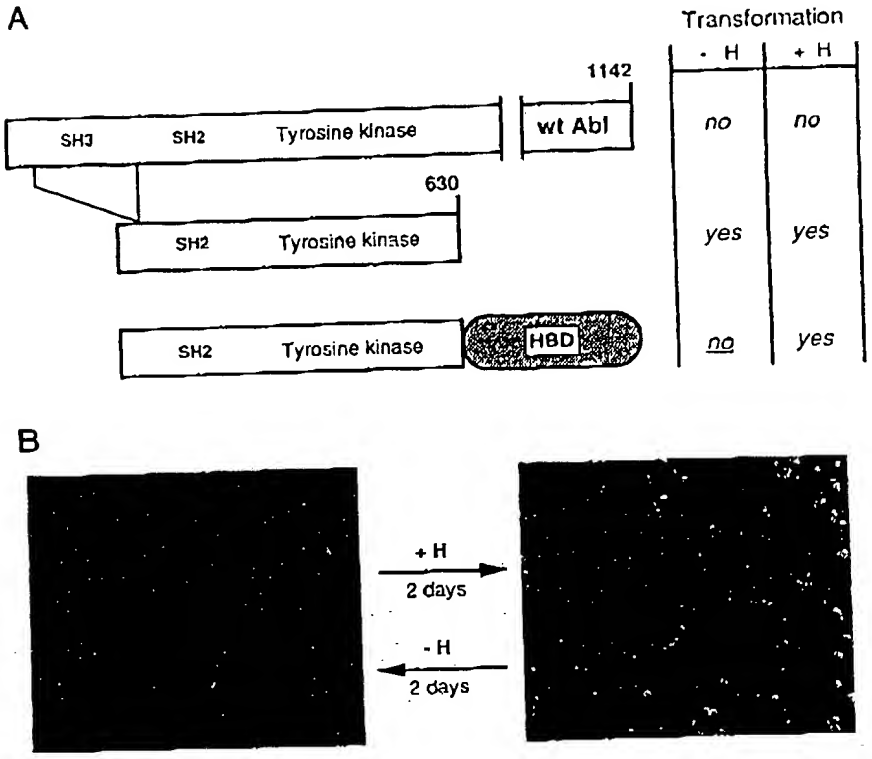

Fig. 2 The Abl tyrosine kinase becomes hormone dependent by fusion to an HBD. (A) Schematic representations of wild-type (wt) c-Abl type IV, an oncogenic derivative (SH3 deletion mutant of c-Abl), and a fusion derivative. SH, Src homology domains; H, steroid hormone (added as indicated in Table III). Oncogenic transformation was evaluated by focus assay and soft agar assay with NIH 3T3 cells (after Jackson *et al.*, 1993). (B) Reversible hormone-dependent morphological transformation. Phase contrast micrographs of a stable cell line transformed with the Abl–HBD fusion derivative. Cells were cultured in the absence (*left*) or presence (*right*) of hormone. The interconversion in both directions is completed in 2 days (Jackson *et al.*, 1993).

1. Generation of Abl-Transformed Cell Lines

To obtain cell lines that are transformed with the Abl fusion protein, there are two alternative protocols. (1) Cells are co-transfected with a Moloney murine leukemia proviral clone (helper virus) and a replication-defective retroviral vector expressing the fusion protein (Jackson and Baltimore, 1989). In co-transfected cells, the helper virus allows replication and packaging of the recombinant virus, which can subsequently infect many neighboring cells with high efficiency. Morphologically transformed cells can be scored based on their ability to form a focus within a monolayer of normal cells (focus assay) or to

form a colony in a soft agar assay (Superti-Furga *et al.*, 1991). The soft agar assay is the more stringent assay for transformation because it requires anchorage-independent growth. (2) An expression vector encoding the fusion protein can also be co-transfected with a plasmid containing a selectable marker. The resulting resistant colonies are heterogeneous with respect to their transformed morphology (in the presence of steroid hormone) because of the variability in the copy number of integrated expression plasmid. Therefore, the oncogenic transformation of such colonies must be confirmed by a soft agar assay.

a. Retroviral Infection

1. Co-transfect 8×10^5 NIH 3T3 cells in a 10-cm dish with 10 μg recombinant retroviral DNA (encoding the fusion protein) and 0.5 μg of the Moloney proviral clone pZAP (Goff *et al.*, 1982) using the calcium phosphate transfection protocol.

2. Maintain the cells in medium containing 0.1 μM 17β-estradiol which is changed every 3 days.

3. Score plates for transformed foci by microscopic inspection.

4. Dislodge isolated transformed foci 15 days after transfection and clone by limiting dilution.

5. Screen independent clones for the hormone-dependence of morphological transformation and, by immunoblotting, for expression of the fusion protein.

6. Further analyze clones by a soft agar assay to test for anchorage-independent growth. The soft agar assay also provides an alternative to cloning by limiting dilution for the isolation of individual colonies.

b. Co-transfection with a Selectable Marker

1. Co-transfect 8×10^5 NIH 3T3 cells in a 10-cm dish with 10 μg expression vector (encoding the fusion protein) and 1 μg plasmid carrying the neomycin resistance gene using the calcium phosphate transfection protocol.

2. Maintain the cells in medium containing 0.1 μM 17β-estradiol and 0.5 mg/ml G418. This medium is changed every 3 days.

3. Pick independent colonies after about 2 wk and test for anchorage-independent growth by soft agar assay in the presence or absence of hormone. Alternatively, all colonies from a plate can be pooled for the soft agar assay.

4. Isolate single colonies that appear exclusively in hormone-containing soft agar plates and expand.

5. Confirm expression of the fusion protein by immunoblotting.

2. Reversibility of Hormone-Dependent Transformation

Regardless of the method of choice for the gene transfer, cell clones are isolated in the transformed state. Morphological transformation is easily recog-

nizable, as shown in Fig. 2B (*right*). Note that Abl-mediated transformation is completely reversible (*left*) (see also Jackson *et al.*, 1993). Reversion to normal morphology is obtained within 2 days by switching off the Abl-HBD fusion protein following the aforementioned "deinduction protocol." It is possible to repeatedly pass from the transformed to the reverted state simply by adding or washing out hormone.

Deinduction experiments reveal yet another facet of Abl: its ability to inhibit cell proliferation. At 48 hr after hormone depletion, not only are cells morphologically reverted, as discussed already, but their growth is inhibited as well. A cytostatic effect of Abl overexpression has been reported by several groups, but until today could only be examined by comparing different Abl derivatives (Jackson and Baltimore, 1989) or the same derivative in different cellular contexts (Renshaw *et al.*, 1992). In contrast, our hormone-regulatable system has the great advantage that the two distinct functions of Abl (transformation versus growth inhibition) can be studied using the same derivative expressed in one cell line. The nature of this inhibitory effect remains to be determined, but fusion proteins can once more be of great help for its elucidation.

C. STE11

Vertebrate steroid receptors can also function in yeast in a ligand-dependent fashion, suggesting that all other components of this particular signal transduction pathway are conserved (Metzger *et al.*, 1988; Schena and Yamamoto, 1988; Mak *et al.*, 1989; McDonnell *et al.*, 1989; Picard *et al.*, 1990c). Steroids are specific inducers with little or no effect on the yeast cell (Tanaka *et al.*, 1989). Furthermore, we were able to demonstrate that the inactivation function of HBD also works in yeast (Louvion *et al.*, 1993). Fusion of the ER HBD to the artificial transcriptional activator GAL4.VP16 subjects its activity to hormonal control. This prompted us to test whether the HBDs of various steroid receptors could regulate a true yeast protein. We chose STE11, a serine/threonine kinase involved in the pheromone signal transduction pathway (Chaleff and Tatchell, 1985; Rhodes *et al.*, 1990).

In the budding yeast *Saccharomyces cerevisiae*, mating is initiated by the binding of mating type-specific peptides (the a- and α-pheromones) to G-coupled receptors. The signal is then propagated through the sequential activation of a series of kinases (STE11, STE7, FUS3, and KSS1), leading finally to the phosphorylation and activation of STE12. STE12 is a transcriptional activator for genes involved in cellular differention and in the specific cell-cycle arrest in late G_1 (for review, see Marsh *et al.*, 1991). The HBDs of ER, GR and MR were fused to STE11 (Fig. 3). Typically, yeast plasmids encoding the STE11 derivatives were introduced into an *ste11*$^-$ strain (Rhodes *et al.*, 1990) by electroporation. STE11-induced growth arrest was then assessed by plating the different clones on agar–media containing α factor, the cognate steroid, or both.

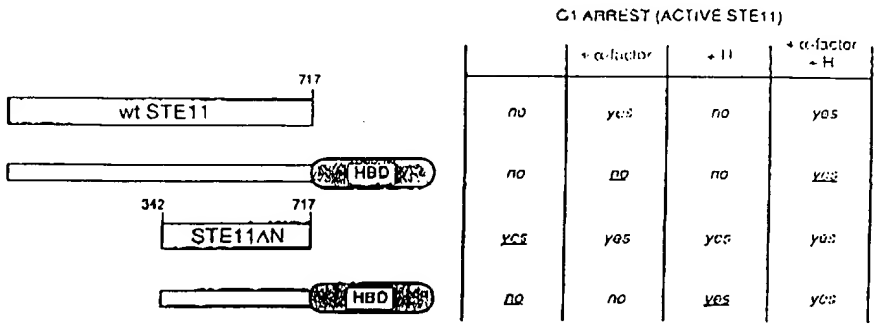

Fig. 3 An STE11-HBD fusion protein is hormone dependent in budding yeast. Wild-type (wt; amino acids 1–717) and N-terminally truncated (STE11ΔN; amino acids 342–717) STE11 were fused in frame with the HBDs of ER, GR, or MR. Episomal expression vectors for these fusion proteins were introduced into a *ste11*⁻ strain by the electroporation technique. Individual clones were then streaked onto agar plates and tested for STE11 activity. Active STE11 effects a cell-cycle arrest (G_1 arrest). α Factor was added at 5 μM and steroid hormones (H) were added as indicated in Table III.

As indicated in Fig. 3, the cells expressing the STE11-HBD fusion proteins are efficiently and specifically growth inhibited only when both α factor and steroid hormone are present, indicating that the unliganded HBD blocks the normal activation pathway. FUS1 is one of the genes activated by the mating response (McCaffrey *et al.*, 1987); therefore, the specificity of the growth arrest was confirmed by measuring the activation of a chromosomally integrated FUS1-*lacZ* reporter gene (Rhodes *et al.*, 1990).

The deletion of the N-terminal regulatory domain of STE11 (derivative STE11ΔN) results in constitutive activation of the kinase (Cairns *et al.*, 1992). Fusion of the HBD to STE11ΔN renders G_1 arrest exclusively dependent on steroid hormone (with no requirement for α factor). As a result, this system can even be transferred to STE11⁺ strains. This example illustrates very nicely how the fusion protein approach can be used to replace a yeast-specific control (such as pheromone regulation) by an HBD-mediated hormonal control.

Acknowledgments

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References

Berthois, Y., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1986). Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. *Proc. Natl. Acad. Sci. USA* **83**, 2496–2500.

Boehmelt, G., Walker, A., Kabrun, N., Mellitzer, G., Beug, H., Zenke, M., and Enrietto, P. J.

(1992). Hormone-regulated v-rel estrogen receptor fusion protein: Reversible induction of cell transformation and cellular gene expression. *EMBO J.* **11**, 4641–4652.

Braselmann, S., Graninger, P., and Busslinger, M. (1993). A selective transcriptional induction system for mammalian cells based on Gal4-estrogen receptor fusion proteins. *Proc. Natl. Acad. Sci. USA* **90**, 1657–1661.

Briegel, K., Lim, K.-C., Planck, C., Beug, H., Engel, J. D., and Zenke, M. (1993). Ectopic expression of a conditional GATA-2/estrogen receptor chimera arrests erythroid differentiation in a hormone-dependent manner. *Genes Dev.* **7**, 1097–1109.

Burk, O., and Klempnauer, K.-H. (1991). Estrogen-dependent alterations in differentiation state of myeloid cells caused by a v-*myb*/estrogen receptor fusion protein. *EMBO J.* **10**, 3713–3719.

Cairns, B. R., Ramer, S. W., and Kornberg, R. D. (1992). Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase. *Genes Dev.* **6**, 1305–1318.

Chaleff, D. T., and Tatchell, K. (1985). Molecular cloning and characterization of the *STE7* and *STE11* genes of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **5**, 1878–1886.

Christopherson, K. S., Mark, M. R., Bajaj, V., and Godowski, P. J. (1992). Ecdysteroid-dependent regulation of genes in mammalian cells by a *Drosophila* ecdysone receptor and chimeric transactivators. *Proc. Natl. Acad. Sci. USA* **89**, 6314–6318.

Daley, G. Q., and Ben-Neriah, Y. (1991). Implicating the Bcr-Abl gene in the pathogenesis of Philadelphia chromosome-positive human leukemia. *Adv. Cancer Res.* **57**, 151–184.

Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J.* **11**, 1025–1033.

Danielian, P. S., White, R., Hoare, S. A., Fawell, S. E., and Parker, M. G. (1993). Identification of residues in the estrogen receptor that confer differential sensitivity to estrogen and hydroxytamoxifen. *Mol. Endocrinol.* **7**, 232–240.

Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. (1989). Chimeras of the MYC oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature (London)* **340**, 66–68.

Fankhauser, C. P., Briand, P.-A., and Picard, D. (1994). The hormone binding domain of the mineralocorticoid receptor can regulate heterologous activities *in cis*. *Biochem. Biophys. Res. Commun.* **200**, 195–201.

Fawell, S. E., Lees, J. A., White, R., and Parker, M. G. (1990). Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell* **60**, 953–962.

Flint, J., and Shenk, T. (1989). Adenovirus E1A protein paradigm viral transactivator. *Annu. Rev. Genet.* **23**, 141–161.

Garabedian, M. J., and Yamamoto, K. R. (1992). Genetic dissection of the signaling domain of a mammalian steroid receptor in yeast. *Mol. Biol. Cell* **3**, 1245–1257.

Goff, S. P., Tabin, C. J., Wang, J. Y.-J., Weinberg, R., and Baltimore, D. (1982). Transfection of fibroblasts by cloned Abelson murine leukemia virus DNA and recovery of transmissible virus by recombination with helper virus. *J. Virol.* **41**, 271–285.

Gronemeyer, H. (1991). Transcription activation by estrogen and progesterone receptors. *Annu. Rev. Genet.* **25**, 89–123.

Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., and Milgrom, E. (1989). Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. *Cell* **57**, 1147–1154.

Hollenberg, S. M., Cheng, P. F., and Weintraub, H. (1993). Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. *Proc. Natl. Acad. Sci. USA* **90**, 8028–8032.

Hope, T. J., Huang, X. J., McDonald, D., and Parslow, T. G. (1990). Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: Mapping cryptic functions of the arginine-rich motif. *Proc. Natl. Acad. Sci. USA* **87**, 7787–7791.

Hope, T. J., Klein, N. P., Elder, M. E., and Parslow, T. G. (1992). *trans*-Dominant inhibition of

human immunodeficiency virus type 1 Rev occurs through formation of inactive protein complexes. *J. Virol.* **66**, 1849–1855.

Ince, B. A., Zhuang, Y., Wrenn, C. K., Shapiro, D. J., and Katzenellenbogen, B. S. (1993). Powerful dominant negative mutants of the human estrogen receptor. *J. Biol. Chem.* **268**, 14026–14032.

Israel, D. I., and Kaufman, R. J. (1993). Dexamethasone negatively regulates the activity of a chimeric dihydrofolate reductase/glucocorticoid receptor protein. *Proc. Natl. Acad. Sci. USA* **90**, 4290–4294.

Jackson, P., and Baltimore, D. (1989). N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-*abl*. *EMBO J.* **8**, 449–456.

Jackson, P., Baltimore, D., and Picard, D. (1993). Hormone-conditional transformation by fusion proteins of c-Abl and its transforming variants. *EMBO J.* **12**, 2809–2819.

Kumar, V., and Chambon, P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55**, 145–156.

Land, H., Parada, L. F., and Weinberg, R. A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (London)* **304**, 596–602.

Louvion, J.-F., Havaux-Copf, B., and Picard, D. (1993). Fusion of GAL4-VP16 to a steroid binding domain provides a tool for gratuitous induction of galactose-responsive genes in yeast. *Gene* **131**, 129–134.

Mak, P., McDonnell, D. P., Weigel, N. L., Schrader, W. T., and O'Malley, B. W. (1989). Expression of functional chicken oviduct progesterone receptors in yeast (*Saccharomyces cerevisiae*). *J. Biol. Chem.* **264**, 21613–21618.

Marsh, L., Neiman, A. M., and Herskowitz, I. (1991). Signal transduction during pheromone response in yeast. *Annu. Rev. Cell Biol.* **7**, 699–728.

McCaffrey, G., Clay, F. J., Kelsay, K., and Sprague, G. F., Jr. (1987). Identification and regulation of a gene required for cell fusion during mating of the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**, 2680–2690.

McDonnell, D. P., Pike, J. W., Drutz, D. J., Butt, T. R., and O'Malley, B. W. (1989). Reconstitution of the vitamin D-responsive osteocalcin transcription unit in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**, 3517–3523.

Metzger, D., White, J. H., and Chambon, P. (1988). The human estrogen receptor functions in yeast. *Nature (London)* **334**, 31–36.

Nevins, J. R. (1990). Adenovirus E1A-dependent trans-activation of transcription. *Sem. Cancer Biol.* **1**, 59–68.

Picard, D. (1993). Steroid-binding domains for regulating the functions of heterologous proteins in *cis*. *Trends Cell Biol.* **3**, 278–280.

Picard, D., and Yamamoto, K. R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J.* **6**, 3333–3340.

Picard, D., Salser, S. J., and Yamamoto, K. R. (1988). A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. *Cell* **54**, 1073–1080.

Picard, D., Khursheed, B., Garabedian, M. J., Fortin, M. G., Lindquist, S., and Yamamoto, K. R. (1990a). Reduced levels of hsp90 compromise steroid receptor action *in vivo*. *Nature (London)* **348**, 166–168.

Picard, D., Kumar, V., Chambon, P., and Yamamoto, K. R. (1990b). Signal transduction by steroid hormones: Nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. *Cell Reg.* **1**, 291–299.

Picard, D., Schena, M., and Yamamoto, K. R. (1990c). An inducible expression vector for both fission and budding yeasts. *Gene* **86**, 257–261.

Pratt, W. B. (1990). Interaction of hsp90 with steroid receptors: Organizing some diverse observations and presenting the newest concepts. *Mol. Cell. Endocrinol.* **74**, C69–76.

Renshaw, M. W., Kipreos, E. T., Albrecht, M. R., and Wang, J. Y. J. (1992). Oncogenic v-Abl tyrosine kinase can inhibit or stimulate growth, depending on the cell context. *EMBO J.* **11**, 3941–3951.

Rhodes, N., Connell, L., and Errede, B. (1990). STE11 is a protein kinase required for cell-type-specific transcription and signal transduction in yeast. *Genes Dev.* **4**, 1862–1874.

Roemer, K., and Friedmann, T. (1993). Modulation of cell proliferation and gene expression by a p53–estrogen receptor hybrid protein. *Proc. Natl. Acad. Sci. USA* **90**, 9252–9256.

Rosenberg, N., and Witte, O. N. (1988). The viral and cellular forms of the Abelson (abl) oncogene. *Adv. Virus Res.* **35**, 39–81.

Ruley, H. E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature (London)* **304**, 602–606.

Rusconi, S., and Yamamoto, K. R. (1987). Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. *EMBO J.* **6**, 1309–1315.

Samuels, M. L., Weber, M. J., Bishop, J. M., and McMahon, M. (1993). Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. *Mol. Cell. Biol.* **13**, 6241–6252.

Schena, M., and Yamamoto, K. R. (1988). Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. *Science* **241**, 965–967.

Schuermann, M., Hennig, G., and Müller, R. (1993). Transcriptional activation and transformation by chimaeric Fos-estrogen receptor proteins: Altered properties as a consequence of gene fusion. *Oncogene* **8**, 2781–2790.

Shenk, T., and Flint, J. (1991). Transcriptional and transforming activities of the adenovirus E1A proteins. *Adv. Cancer Res.* **57**, 47–85.

Smith, D. F., and Toft, D. O. (1993). Steroid receptors and their associated proteins. *Mol. Endocrinol.* **7**, 4–11.

Spitkovsky, D., Steiner, P., Lukas, J., Lees, E., Pagano, M., Schulze, A., Joswig, S., Picard, D., Tommasino, M., Eilers, M., and Jansen-Dürr, P. (1994). Modulation of cyclin gene expression by adenovirus E1A in a cell line with E1A-dependent conditional proliferation. *J. Virol* **68**, 2206–2214.

Superti-Furga, G., Bergers, G., Picard, D., and Busslinger, M. (1991). Hormone-dependent transcriptional regulation and cellular transformation by Fos-steroid receptor fusion proteins. *Proc. Natl. Acad. Sci. USA* **88**, 5114–5118.

Tanaka, S., Hasegawa, S., Hishinuma, F., and Kurata, S. (1989). Estrogen can regulate the cell cycle in the early G1 phase of yeast by increasing the amount of adenylate cyclase mRNA. *Cell* **57**, 675–681.

Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I., and Chambon, P. (1989). The cloned human estrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J.* **8**, 1981–1986.

Umek, R. M., Friedman, A. D., and McKnight, S. L. (1991). CCAAT-enhancer binding protein: A component of a differentiation switch. *Science* **251**, 288–292.

Vegeto, E., Allan, G. F., Schrader, W. T., Tsai, M.-J., McDonnell, D. P., and O'Malley, B. W. (1992). The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of the human progesterone receptor. *Cell* **69**, 703–713.

Wrange, Ö., Eriksson, P., and Perlmann, T. (1989). The purified activated glucocorticoid receptor is a homodimer. *J. Biol. Chem.* **264**, 5253–5259.

Wrenn, C. K., and Katzenellenbogen, B. S. (1993). Structure-function analysis of the hormone binding domain of the human estrogen receptor by region-specific mutagenesis and phenotypic screening in yeast. *J. Biol. Chem.* **268**, 24089–24098.

Wright, A. P., and Gustafsson, J.-Å. (1992). Glucocorticoid-specific gene activation by the intact human glucocorticoid receptor expressed in yeast. Glucocorticoid specificity depends on low level receptor expression. *J. Biol. Chem.* **267**, 11191–11195.

Wright, A. P., Carlstedt-Duke, J., and Gustafsson, J.-Å. (1990). Ligand-specific transactivation of gene expression by a derivative of the human glucocorticoid receptor expressed in yeast. *J. Biol. Chem.* **265**, 14763–14769.

Yamamoto, K. R., Godowski, P. J., and Picard, D. (1988). Ligand regulated nonspecific inactivation of receptor function: A versatile mechanism for signal transduction. *Cold Spring Harbor Symp. Quant. Biol.* **53**, 803–811.

Ylikomi, T., Bocquel, M. T., Berry, M., Gronemeyer, H., and Chambon, P. (1992). Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.* **11**, 3681–3694.

CERTIFICATE OF MAILING

I hereby certify that this paper and every paper referred to therein as being enclosed is being deposited with the U.S. Postal Service as first class mail, postage prepaid, in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, DC 20231, on August 31, 2000 (Date of Deposit)

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Docket No: **0630/0D532**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Lisa A. **NEUHOLD** et al.

Serial No.: 08/994,689 Art Unit: 1633

Filed: December 19, 1997 Examiner: M. Wilson

For: **TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE**

AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

In response to the Office Action dated March 1, 2000, please consider the following amendments and remarks. This amendment is accompanied by (1) a Petition for Extension of Time for Three Months, to and including September 1, 2000, accompanied by the required fee, and (2) a Second Declaration of Lisa A. Neuhold, Ph.D. Under 37 C.F.R. § 1.132 (Second Neuhold Declaration).

IN THE CLAIMS:

Please cancel claims 1, 2, 4-9, 11, 12, 15-17, and 22-28 without prejudice.

Please add the following claims:

--29. A transgenic non-human mammal or progeny thereof whose somatic and germline cells contain, in stably integrated form,

(a) a first coding sequence encoding an enzymatically active matrix degrading enzyme (MDE) that degrades an extracellular matrix component, wherein expression of the first coding sequence is under control of a regulatable promoter that is responsive to a transcriptional repressor or activator polypeptide; and

(b) a second coding sequence encoding the transcriptional repressor or activator polypeptide, wherein expression of the second coding sequence is under control of a chondrocyte tissue-specific promoter;

wherein expression of the MDE by chondrocytes is repressed throughout embryonic, fetal, and early postnatal development, and activation of MDE expression results in a phenotypic change characteristic of osteoarthritis.

30. The transgenic mammal of claim 29, wherein the MDE is a matrix metalloproteinase selected from the group consisting of MMP-1, MMP-3, MMP-8, and MMP-13.

31. The transgenic mammal of claim 29, wherein the MDE is constitutively enzymatically active without proteolytic processing.

32. The transgenic mammal of claim 31, wherein the MDE is a constitutively enzymatically active MMP-13 variant.

33. The transgenic mammal of claim 32, wherein the MMP-13 variant has a

sequence of ID NO:1 or SEQ ID NO:21.

34. The transgenic mammal of claim 29, wherein the mammal is selected from the group consisting of a mouse, a rat, and a rabbit.

35. The transgenic mammal of claim 29, wherein the mammal is a mouse.

36. The transgenic mammal of claim 29, wherein the transcriptional repressor or activator polypeptide is a repressor polypeptide.

37. The transgenic mammal of claim 36, wherein the repressor polypeptide is a tetracycline repressor polypeptide.

38. The transgenic mammal of claim 37, wherein the regulatable promoter comprises a tet07 sequence.

39. The transgenic mammal of claim 38, wherein the regulatable promoter has the sequence depicted in SEQ ID NO:2.

40. The transgenic mammal of claim 29, wherein the chondrocyte tissue-specific promoter comprises sequences from a Type II collagen promoter.

41. The transgenic mammal of claim 29, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

42. A transgenic mouse or rat, or progeny thereof, whose somatic and germline cells contain, in stably integrated form,

(a) a first coding sequence encoding a constitutively enzymatically active matrix metalloproteinase (MMP) that cleaves Type II collagen, wherein expression of the first coding sequence is under control of a tetracycline-regulatable promoter; and

(b) a second coding sequence encoding a tetracycline repressor polypeptide that binds to the tetracycline-regulatable promoter, wherein expression of the second coding sequence is under control of a chondrocyte tissue-specific promoter;

wherein expression of the MMP by chondrocytes is repressed throughout embryonic, fetal, and early postnatal development, and activation of MMP expression results in a phenotypic change characteristic of osteoarthritis in the transgenic mouse or rat.

43. The transgenic mouse or rat of claim 42, wherein the collagenase is constitutively enzymatically active MMP-13, the tetracycline regulatable promoter is a tet07 promoter, the tetracycline repressor polypeptide is a tTA polypeptide, and the chondrocyte tissue-specific promoter comprises sequences from a Type II collagen promoter.

44. The transgenic mouse or rat of claim 43, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

45. A method for producing a phenotypic change characteristic of osteoarthritis in a transgenic mammal of claim 29, which method comprises activating MDE expression in the transgenic mammal after embryonic, fetal, and early postnatal development.

46. The method according to claim 45, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

47. A method for producing a phenotypic change characteristic of osteoarthritis in the transgenic mammal of claim 37, which method comprises maintaining the transgenic mammal on tetracycline or a tetracycline analog during embryonic, fetal, and early postnatal development, and activating the MDE expression by withholding the tetracycline or tetracycline analog after embryonic, fetal, and early postnatal development.

48. The method according to claim 47, wherein the tetracycline analog is doxycycline.

49. The method according to claim 47, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

50. A method for producing a phenotypic change characteristic of osteoarthritis in the transgenic mouse or rat of claim 42, which method comprises maintaining the transgenic mouse or rat on tetracycline or a tetracycline analog during embryonic, fetal, and early postnatal development, and activating the collagenase expression by withholding the tetracycline or tetracycline analog after embryonic, fetal, and early postnatal development.

51. The method according to claim 50, wherein the tetracycline analog is

doxycycline.

52. The method according to claim 50, wherein the phenotypic change characteristic of osteoarthritis is selected from the group consisting of loss of proteoglycan, cleavage of type II collagen, gross observations of changes in joint function, joint space narrowing, collagen degradation, destruction of cartilage, changes in growth plate morphology, fibrillation and loss of articular cartilage, osteophyte formation, and combinations thereof.

53. A method for evaluating a composition to counteract a phenotypic change characteristic of osteoarthritis, which method comprises:

(a) administering the composition to the transgenic mammal of claim 29 in which a phenotypic change characteristic of osteoarthritis has been produced by activation of MDE expression after embryonic, fetal, and early postnatal development of the transgenic mammal;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mammal to which the composition was administered relative to a control mammal in which MDE expression was activated without administering the composition,

wherein any difference in the nature or extent of the phenotypic change, or any difference in the time required for the phenotypic change to develop, indicates the potential of the composition to counteract the phenotypic characteristic of osteoarthritis.

54. A method for evaluating a composition to counteract a phenotypic characteristic of osteoarthritis, which method comprises:

(a) administering the composition to the transgenic mammal of claim 37 in which a phenotypic change characteristic of osteoarthritis has been produced by activating MDE expression by withholding tetracycline or a tetracycline analog after embryonic, fetal, and early postnatal development of the transgenic mammal;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mammal to which the composition was administered relative to a control mammal in which MDE expression was activated without administering the composition,

wherein any difference in the nature or extent of the phenotypic change, or any difference in the time required for the phenotypic change to develop, indicates the potential of the composition to counteract the phenotypic characteristic of osteoarthritis.

55. A method for evaluating a composition to counteract a phenotypic change characteristic of osteoarthritis, which method comprises:

(a) administering the composition to the transgenic mouse or rat of claim 42 in which a phenotypic change characteristic of osteoarthritis has been produced by activating collagenase expression by withholding tetracycline or a tetracycline analog after embryonic, fetal, and early postnatal development of the transgenic mouse;

(b) monitoring the phenotypic change; and

(c) comparing the extent of the phenotypic change in the mouse or rat to which the composition was administered relative to a control mouse or rat in which collagenase expression was activated without administering the composition,

wherein any difference in the nature or extent of the phenotypic change, or any difference in the time required for the phenotypic change to develop, indicates the potential of the composition to counteract the phenotypic characteristic of osteoarthritis.--

REMARKS

Applicants acknowledge the courtesy shown them by the Examiner, the Examiner's Supervisor John Leguyader, and Biotechnology Practice Specialist Brian Stanton during the interview with co-inventor Lisa A. Neuhold, Ph.D., American Home Products in-

house patent counsel Elizabeth M. Barnhard, and the undersigned attorney for applicants on May 17, 2000, during which the rejections for lack of enablement were discussed. The Second Neuhold Declaration states, in writing, and where appropriate expands upon, the points Dr. Neuhold made during the interview, including exhibits presented at the interview.

The pending claims have been canceled and new claims 29-55 have been added to more particularly point out and distinctly claim that which applicants regard as the invention. The new claims are fully supported by the claims as filed and by the specification. In particular, support for new claims 29-55 is set forth in the following table:

New Claims	Support
29.	Claim 1 as filed; p. 27, l. 9 (coding sequence); at p. 2, l. 4 and p. 5, ll. 19-20 (enzymatically active matrix degrading enzyme (MDE) that degrades an extracellular matrix component); p. 6, ll. 11-12 (regulatable promoter); p. 13, ll. 6-8 (promoter that is responsive to a transcriptional repressor or activator polypeptide); p. 37, l. 1, p. 6, ll. 4-5, p. 13, ll. 3-4 (chondrocyte tissue-specific promoter); p. 13, l. 1, p. 16, ll. 12-15 (expression repressed throughout embryonic, fetal, and early postnatal development); p. 6., l. 5-6, p. 18, ll. 9-11 (MDE expression results in a phenotypic change characteristic of osteoarthritis).
30.	p. 2, Table 1 and p. 5, ll. 23-24.
31.	p. 12, l. 8.
32.	p. 12, l. 13.
33.	claim 7 as filed; p. 12, ll. 16-18.

34.	claim 8 as filed.
35.	claim 9 as filed.
36.	p. 13, l. 10.
37.	p. 14, l. 21.
38.	claim 11 as filed; p. 14, ll. 21-22
39.	claim 12 as filed
40.	claim 15 as filed; p. 16, l. 2.
41. (also 44, 46, 49, and 52)	p. 18, ll. 13-15; p. 19, ll. 11-18; p. 21, ll. 5-6.
42.	p. 27, l. 9 (coding sequence); p. 12, ll. 8-12 (constitutively active MMP); p.3, ll. 4-5, p. 11, ll. 5-6 (degrades Type II collagen) p. 6, ll. 11-12; p. 13, ll. 6-8 and 19; (tetracycline regulatable promoter); p. 13, l. 19 (tetracycline repressor polypeptide); p. 37, l. 1; p. 6, ll. 4-5; p. 13, ll. 3-4 (chondrocyte tissue-specific promoter); p. 13, l. 1 and p. 16, ll. 12-15 (expression repressed throughout embryonic, fetal, and early postnatal development); p. 18, ll. 9-11 (MMP expression results in phenotypic change characteristic of osteoarthritis).
43.	claim 16 as filed; p. 17, l. 24 to p. 18, l. 2.

45. (also 47 and 50)	claims 22-24 as filed; p. 18, ll. 16-21.
48. (also 51)	p. 41, l. 6.
53-55	claims 25-27 as filed; p. 20, l. 13 to p. 21, l. 2.

SUMMARY OF POINTS

Based on the grounds for rejection in the outstanding Office Action and the discussion at the interview, applicants submit that there are no issues with respect to patentability of the specific transgenic mice exemplified in this application. However, as discussed at the interview, such a limited claim scope denies the protection to which this discovery is entitled: any transgenic animal that expresses an enzymatically active matrix degrading enzyme under control of an inducible promoter and a tissue-specific promoter, and, when induced to express the enzymatically active matrix degrading enzyme, develops phenotypic changes characteristic of osteoarthritis. Applicants wish to emphasize the following points in support of patentability of the invention as presently claimed:[1]

- Enzymatically active matrix degrading enzymes that degrade extracellular matrix components are well known. Applicants have established this in the file history of the application as follows:

  Specification: pages 2-3; pages 11-12.

  Additional evidence: Second Neuhold Declaration, paragraph 8.

- Expression of coding sequences under control of regulatable promoters that are

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[1] Applicants refer herein to the Declaration of Lisa A. Neuhold, Ph.D. Under 37 C.F.R. § 1.132 filed April 6, 1999 (the Neuhold Declaration), the preliminary Amendment filed by hand on February 18, 2000 (the Preliminary Amendment) and the accompanying Second Neuhold Declaration.

responsive to a transcriptional repressor or activator polypeptide in transgenic animals is well known. Applicants have established this in the file history of this application as follows:

Specification: pages 12-15 and pages 16-18.

Additional Evidence: Exhibits A, B, C, D, and E to Preliminary Amendment; Exhibit1 1 attached hereto; Second Neuhold Declaration, paragraph 6.

- Tissue specific expression of transgenes in transgenic animals is well known. Applicants have established this in the file history of this application as follows:

  Specification: pages 15-16 and 17-18.

  Additional Evidence: Neuhold Declaration, paragraph 6; Second Neuhold Declaration, paragraph 7 and Tab 2.

- Transgenic nonhuman mammals, particularly mice, rats, and rabbits, are well known and prepared routinely by ordinary research scientists at the time this invention was made. It is also a known and accepted that, as with every other experimental system in biology such as cloning and hydridomas, not every transgenic embryo will yield a transgenic animal with the desired characteristics, but that routine screening and selection techniques will provide such an animal as claimed. Applicants have established this extensively in the file history of this application as follows:

  Specification: pages 22-26.

  Additional Evidence: Second Neuhold Declaration, paragraph 9 and Tab 4.

- Phenotypic characteristics of osteoarthritis (degenerative bone disease), as developed in the transgenic animals of the invention are well-known. Applicants have established this in the file history of the application as follows:

  Specification: pages 18-20, 21 and 45.
  Additional Evidence: Neuhold Declaration, paragraphs 6, 11, 13 and 14; Exhibits F, G, H, and I to the Preliminary Amendment; Second Neuhold Declaration, paragraph 10.

- The totality of the evidence of record in the file history of this application establishes that the claimed transgenic mammals, particularly the claimed transgenic mice and rats, can be generated and induced to develop one or more phenotypic characteristics of osteoarthritis. These transgenic animals thus serve as useful models for studying the progression and evaluating therapies for this disease. Applicants have established these features of the invention throughout the file history of the application as set forth and as follows:

  Specification: pages 1, 5-7, 18-20, 21 and 45.
  Additional Evidence: Neuhold Declaration, paragraph13; Second Neuhold Declaration, paragraph 11.

THE SPECIFICATION ENABLES THE CLAIMED INVENTION

The Examiner has rejected claims 1, 2, 4-9, 11, 12, 15-17, and 22-28 under 35 U.S.C. § 112, first paragraph, contending that while being enabling only for MMP13* (SEQ ID NO:1) linked to tet07 promoter + tet repressor and VP16 activator linked to type II collagen promoter where a mouse is given tetracycline until adulthood, the specification does not provide enablement for a mammal.

Applicants respectfully traverse this rejection. For the reasons advanced above in the accompanying Second Neuhold Declaration, the specification enables claims to mammals. In particular, ". . . contrary to the examiner's assertions, as of 1996 creation of transgenic mammals required no more than ordinary technical efforts – indeed, technical efforts with shortcomings that are readily overcome" (Neuhold Declaration, paragraph 9). All of these techniques are set forth in the specification at pages 22-26. For the reasons discussed in greater detail below, the state of the art at the time this invention was made was much farther advanced than the Examiner allows, and the Examiner's contention is incorrect.

The Examiner further contends that the specification does not teach how to get phenotype with any other regulating system. As discussed during the interview and set forth in the accompanying Second Neuhold Declaration at paragraph 6, the regulatable expression systems described in the specification are well established and well known in the art. As set forth above, applicants have submitted numerous references further supporting enablement of this aspect of the invention. The Examiner appeared to agree with this position at the interview. Accordingly, this basis for the rejection is overcome and should be withdrawn.

The Examiner also contends that the specification does not teach how to get other phenotypes. While Applicants are uncertain what exactly the examiner means, the record as set forth above and the Second Neuhold Declaration (paragraphs 10 and 11) firmly establish that the

transgenic animal of the invention, upon expression of the matrix degrading enzyme, develops a phenotypic change characteristic of osteoarthritis.

The Examiner further states that it is not clear from reading the specification, whether readministration of tet would cause reversion back to wild type. This issue has been addressed in Preliminary Amendment (page 15, first full paragraph). In short, it simply does not matter. This concern is misplaced, as, according to the terms of the invention, it is induction of MDE expression (for example, in a tet-repressor regulated system, by cessation of doxycycline administration) that leads to the claimed phenotype. Thus, this basis for rejecting the claims is unfounded and should be withdrawn.

The Examiner states that applicants should point to specific activator/repressor and regulatory elements in the specification and correlate them to the VP16/TET/TET REPRESSOR system of the examples. As discussed above, this is done in the specification (see pages 12-15 and 16-17). The Second Neuhold Declaration further establishes that regulatable expression systems are well established in transgenic animals (paragraph 6). The Examiner appeared to agree with this position at the interview. Accordingly, this basis for the rejection is overcome and should be withdrawn.

The Examiner contends that it is not clear whether other extracellular matrix degrading enzymes would achieve the claimed phenotype. Applicants respectfully disagree. The specification sets forth a plethora of matrix degrading enzymes (pages 2-3); the Examiner has provided no evidence or documentation to substantiate doubts that other MDEs would achieve this phenotype. The Examiner has the burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). MPEP § 2164.04. The Examiner has clearly failed to meet his burden here. In contrast, Applicants have more than met theirs: in addition to the express disclosure of the specification (see pages 2-3 and 11-12), they have further addressed this issue in the interview and by Rule

132 Declaration (*see* the Second Neuhold Declaration, paragraph 8). Thus, this rejection is overcome and should be withdrawn.

The Examiner asserts that the invention is unpredictable with regard to phenotype. However, the evidence of record in the specification (pages 41-44), the Neuhold Declaration (paragraphs 9, 11, 13, 14, and 15), and the Second Neuhold Declaration (paragraphs 10 and 11) firmly establish that the transgenic animals of the invention demonstrate the claimed phenotypic change, *i.e.*, one or more characteristics of osteoarthritis.

The Examiner contends that the specification does not teach that expression can be turned on any time during early post natal development to get phenotype. For the reasons discussed above in connection with regulatable expression, Applicants believe that this basis for rejecting the claims is moot.

The Examiner also asserts that non-mutated MMP genes are not enabled. As these genes are extremely well known in the art (as agreed at the interview), which is specifically established on pages 2-3 of the specification, Applicants submit that this rejection is obviated and should be withdrawn.

The Examiner further contends that the specification is only enabled for MMP13* and exemplified phenotype. For the reasons set forth above, Applicants submit that the specification broadly enables transgenic mammals that express any MDE in a tissue specific, temporally regulated fashion. Furthermore, as the exemplified phenotype is a phenotypic change characteristic of osteoarthritis (Second Neuhold Declaration, paragraphs 10 and 11), the specification clearly enables this aspect of the claimed invention as well. Thus, these bases for rejecting the claims are obviated and should be withdrawn.

The Examiner contends that the specification is not enabled for any joint specific promoter. As discussed during the interview and set forth in the Second Neuhold Declaration (paragraph 7), the specific promoter employed to achieve tissue specific expression does not make any difference, as one of ordinary skill in the art would readily appreciate. A number of

issued patents that cover transgenic animals establish tissue-specific expression is sufficiently enabled for expression of a transgene, because the actual tissue specific promoter is usually of no moment. Moreover, it is proper in a patent for a transgenic animal to claim the promoter by virtue of its tissue specificity rather than identity. *See* U.S. Patent Nos. 5,625,124 (claim 1: "gut epithelial cell specific promoter"); 5,880,327 (claim 1: "a mammary-gland specific promoter"); 5,917,123 (claim 1: "a cardiac-specific regulatory region"); 6,028,245 (claim 1: "a promoter that drives expression of the transgene in skin") (attached as Exhibit 2). In view of the foregoing, the Examiner's basis for this rejection is obviated and should be withdrawn.

The Examiner cites references detailing expression in other mammals as support for argument that the specification does not enable non-human mammals besides mouse. Applicants respectfully take issue with these citations on two grounds. First, they support the opposite conclusion: that the claimed transgenic animals are enabled. Second, these references do not adequately support the rejection. Applicants submit that the Examiner has not carried this burden where the support (1) generally refers to the generic technology; (2) addresses questions related to economics and commercialization, not § 112, first paragraph; (3) contains no information specifically relevant to the claimed invention; and (4) is out of date (none of the references cited by the Examiner have publication dates later than 1996; one was published in 1988; in a rapidly evolving field such as transgenic animals, only the most current references from the time the invention was made have any bearing). *Cf. In re Goodman*, 29 USPQ2d 2010 (Fed. Cir. 1993) (applying references directly addressing the claims in issue to establish lack of enablement).

In particular, the Wall reference reports that 6000 papers describe transgenic animals, mostly mice, to answer research questions (pages 58, 60, and 61). Wall states that ". . . genes can . . . be modified to function very differently than they do in their native form (gene products, tissue specificity, and timing of expression can be altered)" (page 58). In other words, Wall specifically states that the features of Applicants' invention can be achieved. Wall does

concede that transgenic farm animals are costly (mostly because it takes many attempts to yield the desired transgenic animal) (see page 60), however, economic issues are irrelevant to enablement. How is it possible that a reference acknowledging such an abundance of research papers on transgenic animals, manipulation of expression, and at least 1% efficiency of obtaining the desired transgenic animal (much higher, one might add, than the likelihood of obtaining a desired monoclonal antibody or even cloning a gene) calls into question enablement of this invention? On the contrary, Applicants might very well (and here do) cite such a reference to support the routine nature of generating experimental transgenic animals for disease models.

The Ebert reference (from 1988) reports success ("Transgenic pigs carrying this fusion gene had elevated levels of circulating human somatrotropin"; page 277). Applicants submit that the presence of failures is irrelevant in the face of success. The entire history of biology is one of selecting and screening for successes from the much more abundant failures. *See In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988) (enablement of broad monoclonal antibody claims despite the large number of trials necessary to obtain the operative antibody).

Mullins and Mullins, like Wall, report that transgenic technology, including ES technology, is well established (page S37). Time and cost, issues irrelevant to enablement, limit the desirability of pronuclear injection in larger mammals. No matter, as pointed out in the specification, ES technology is an alternative. In any event, the fact that pronuclear injection is less efficient, and therefore economically undesirable, fails to establish that it does not work. On the contrary, nothing in Mullins and Mullins supports such a conclusion. In any event, this paper reports on a number of successful non-murine transgenic animal models (see page S38).

Finally, the Overbeck reference shows that different transgenic animals will demonstrate different levels of expression. Regulatory sequences help avoid variability (see page 97), but this makes little difference: variability ranges from one extreme to another, from no phenotypic change to the desired change. The Examiner contends that this establishes

unpredictability. Applicants disagree. This establishes predictability of two things: there will be failures, and there will also be successes. By selecting the successes, which is routine, one achieves the desired transgenic animals. Indeed, applicants themselves had failures, among which successful animals were obtained (see page 43 of the specification).

In short, the Examiner's grounds for rejection are in error given the advanced state of the art, including general recognition of enablement of transgenic animals (irrespective of whether or not they are cost effective), widespread knowledge of regulatable expression systems, the understanding in the art of tissue-specific expression, and the number of well known extracellular matrix degrading enzymes from which to choose. The present invention is broadly enabled, and the Examiner has not met his burden of challenging enablement with reasonable evidence. Accordingly, the rejection under 35 U.S.C. § 112, first paragraph is in error and should be withdrawn.

CONCLUSION

Applicants respectfully request unto the foregoing amendments and remarks in the file history of this application. In view of the foregoing amendments and remarks, applicants submit that the claims meet all the statutory requirements for patentability. If the Examiner has any other concerns, he is invited to contact the undersigned by telephone. Allowance of the claims is earnestly solicited.

Respectfully submitted,

Date: August 31, 2000

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